THE UNIVERSITY OF ALBERTA

T Cell Regulation of the Induction of Cytotoxic T Cells

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled T Cell Regulation of the Induction of Cytotoxic T cells submitted by Abdul R. Al-Adra in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Immmunology.

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Abstract

Thymus-derived lymphocytes (T cells) participate in a variety of immunological effector functions. They are responsible for the generation of the lytic activity in a mixed lymphocyte culture, the development of delayed-type hypersensitivity and the rejection of allografts. T cells also play a key role in a complex network of immunoregulatory interactions which are both positive (helper effect) and negative (suppressor effect) in character. These observations raise one of the most important questions in cellular immunology. Namely: Does this diversity of function reflect heterogeneity in the T cell population? The present studies aim to identify the biological properties and the physical characteristics of the helper, suppressor and cytotoxic T cells.

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CHAPTER I

Regulatory activity of thymus-derived (T) cells

A. Introduction

Evidence to date suggests that T cells play a crucial regulatory role in the induction of both humoral and cell-mediated immune responses. This regulatory activity could be viewed in algebraic terms as being either positive or negative. Positive regulation leads to induction and negative regulation results in unresponsiveness or immunosuppression.

The cooperative activity (positive regulation) of T cells involved in the development of antibody and cell-mediated immune responses was the first regulatory function attributed to T cells (1). T cells that function in this manner came to be known as "helper" T cells. The importance of helper T cell activity in the production of antibody responses was best dramatized in the hapten-carrier cell transfer studies of Mitchison (2-4). He demonstrated that the interaction of T cells with antigenic determinants on the carrier molecule led to the production of anti-hapten antibodies by B cells. This observation has been confirmed and extended by Paul (5) and Katz and Benacerraf (6). Similarly, helper activity is also required for the generation of cell-mediated immunity. Cantor et al. (7) demonstrated that two distinct population of T cells, termed

effector and amplifier T cells, interacted synergistically in the development of graft vs host reactions in the mouse. Similar observations were reported by Wagner (8), Cohen and Howe (9), Pilarski (10) and Bach et al (11). More recently, cooperative interaction between thymocytes and in vitro cultured splenic T cells has been described for the generation of cytotoxic allograft responses in vitro (12).

Negative regulatory mechanisms or immunosuppression, are part of a homeostatic and self-monitoring immune system. That is, recognition of antigen not only stimulates the proliferation and diferentiation of responder cells but also stimulates the generation of specific immunosuppressive effects which appear to inhibit the induction of immune responses or to modify an ongoing response. These immunosuppressive effects could be mediated by clone inactivation or deletion, negative feedback by antibody or antigen-antibody complexes, active suppression by cells, and anti-receptor antibody. Clonal deletion was postulated by Burnet (13) as a working hypothesis for the maintainance of self-tolerance. He suggested that newly differentiated immunocompetent cells were destroyed, or rendered incapable of multiplication, when they recognized and reacted with antigenic determinants of self-components. This theory has recently become a subject of debates and various criticisms by many immunologists who favor a mechanism whereby specific immune cells exist whose sole function is to maintain the unresponsive state against self-components. Further

discussion of this subject is beyond the scope of this thesis and therefore will not be pressed here. Also, arguments that antibody, antibody-antigen complexes and anti-receptor antibody have inhibitory effects has been extensively reviewed by Uhr and Moller (14), Playfair (15), Rajewsky and Eichmann (16), and Diener (17).

Evidence which suggests that immunosuppressive effects could be attributed to supressor T cells has accumulated only recently. The concept of suppressor T cell activity in immune responses was introduced, and vigorously championed, by Gershon and Kondo (18,19). They observed that the unresponsiveness of B cells in thymectomized, lethally irradiated and bone marrow reconstituted adult CBA mice was dependent on the presence of T cells. The pretreatment of bone marrow reconstituted mice with SRBC in the absence of T cells had no significant effect on the ability of thymocytes to reconstitute the anti-SRBC antibody response. On the other hand, mice which had been reconstituted with a small number of thymocytes at the time of bone marrow reconstitution (prior to the antigen injections) were markedly impaired in their ability to make anti-SRBC antibodies. Following these studies, a number of suppressor cell phenomena were described. In each case, a common property of suppressor cells is that one cell population will inhibit the response, or function, of a second cell population in an *in vivo* cell transfer or *in vitro* experiment. The majority of the inhibitory cell populations

observed are anti-theta serum-sensitive, confirming the T cell dependence of suppressor activity. Furthermore, depending on the mode of action, the suppressive effect can be either antigen specific or nonspecific.

The fact that T lymphocytes function as effector cells in cell-mediated immunity and can suppress, as well as help, immune responses raises one of the most important questions in cellular immunology; namely, are these functions mediated by one subset, or different subsets, of T cells? In other words, does this diversity of function reflect heterogeneity in the T cell population? A convenient model for exploring the nature of the division of labor in the T cell compartment is the _in vitro_ generation of cytotoxic T cells. This system is very well characterized in the literature (20-22). Furthermore, T cells which can either help or suppress the induction of cytotoxic responses have also been reported (23-30). However, the nature and mechanism of the regulatory interactions which lead to the induction of immune effector functions in these systems remain somewhat obscure. The present studies aim to characterize the relationship between suppressor cells and helper cells and the relationship between suppressor cells and cytotoxic T cells.

B. Review of the Literature

1. A direct approach for the identification of T cell subclasses.

The characterization of a particular cell type depends on the tools available for the isolation and the identification of this cell type as defined by its function. The Ly alloantisera are some of the most useful tools which allow separation and characterization of distinct subclasses of T cells, and permit definitive studies of the cellular mechanisms underlying T cell regulation. These alloantisera, originally developed by Boyse and colleagues (31,32), define a panel of cell surface markers, called Ly antigens, found on lymphoid cells. Several of these Ly antigens have been identified. Some of these antigens are exclusively expressed on T cells, some on B cells and others on B and T cells. This review will concentrate on those antigens that are found on T cells and I shall adhere to the current terminology in describing them.

Originally, Ly 1, Ly 2 and Ly 3 specificities were described. Genetic studies have shown that each of these specificities is determined by a genetic locus and each locus is expressed as one of two alternative alleles. The Ly 1 alleles are Ly 1.1 and Ly 1.2 which code for the Ly 1.1 and Ly 1.2 antigens, respectively. Likewise, same terminology applies for the Ly 2 and Ly 3 loci. The Ly 1

locus has been located on chromosome 19 and the Ly 2 and Ly 3 loci are closely linked on chromosome 6 (33). Most recently, new Ly determinants have been identified, Ly 5 (34), Ly 6 and Ly 7 (35-37). Ly 5 appears to be restricted to T lymphocytes whereas Ly 6 and Ly 7 seem to be more widely distributed (36,37). However, linkage studies thus far have not yet identified the chromosomal loci of these specificities.

Much excitement has developed recently in view of accumulated evidence that the selective expression of Ly antigens on T lymphocytes is associated with the immunological function of these lymphocytes. Using alloantisera raised against these specificities, Cantor and Boyse (38) suggested that on the basis of the distribution of the Ly antigens, thy 1+ cells can be further divided into three subclasses as follows: (a) Ly 1,2,3+, (b) Ly 1+, and (c) Ly 2,3+. Ontogenic analysis revealed that while Ly 1+ and Ly 2,3+ cells are resistant to the short-term effects of adult thymectomy, Ly 1,2,3+ cells are sensitive to these effects. This suggests that Ly 1,2,3+cells develop earlier in ontogeny than Ly 1+ and Ly 2,3+ cells, or are short-lived. Furthermore, the authors demonstrated that depletion of Ly 1+ cells abolished the subsequent helper activity to SRBC in adoptive syngeneic irradiated hosts. On the other hand, depletion of Ly 2,3+ cells abolished the generation of cytotoxic lymphocytes both *in vivo*, in irradiated F1 hosts, and *in vitro* in 5-day MLC. The

conclusion from these studies and others (as will be discussed below) is that the Ly antigens could serve to identify different subclasses of T cells where each subclass displays a unique set of biological properties and immune functions.

2. Evaluation of the Ly system

The anti-Ly antisera currently most used are produced in congenic mice. The B6 mouse is preferred to any other strain because a larger number of congenic lines are based on B6. This provides the investigator with a greater selection of stringent serological controls to be included for the evaluation and identification of the anti-Ly antiserum in question (39). The only disadvantage of this system is that immunization between congenic partner strains, i.e. strains which are genetically identical to B6 except for the Ly allele in question fails to give rise to Ly antibody (39). This phenomenon, however, is not unique to the Ly series. Shen et al. (39) demonstrated that anti-TL (thymus leukemia antigen) also cannot be produced under the same circumstances. It is not yet known what mechanism could account for this observation, though a "carrier effect" mechanism of the type described by Schiermann and McBride has been implicated (39). Alternatively, F1 hybrid recipients were used to produce the required anti-Ly specificity. For example, immunization of (BALB/c x B6)F1 recipients with thymocytes from B6-Ly 1.1 donors to produce

anti-Ly 1.1 antibodies was superior to immunization of B6-Ly 1.2 congenic mice with thymocytes from the same donor (39). Since, at least in the hands of these authors, Ly antisera are prepared by "noncongenic" immunization, the use of stringent serological controls is imperative (see below).

Another characteristic feature of the Ly immunizations is the large amount of irrelevant antibodies which are often produced. In one instance, recipients of allogeneic thymocytes compatible with the donor for all known serologically demonstrable surface antigens produced virtually nothing but anti-thymocyte autoantibodies. This antiserum was cytotoxic for thymocytes of all mouse strains tested and retained little or no activity after absorption with syngeneic thymocytes (39). Other contaminating antibodies might interfere at the level of the complement-mediated, antibody-dependent cytotoxicity assay. In this assay, detection of surface antigens depends on the ability of the antibody recognizing the antigen to bind and fix complement. In this respect, antibodies of the IgG2 and IgM classes are effective whereas IgG1 antibodies are not (40). Mathieson et al. reported that most of the presently available, highly cytotoxic pools of anti-Ly antisera contain considerable amounts of non-cytotoxic IgG1 anti-Ly antibodies (40). The presence of such specificities could block effective complement-mediated cytotoxicity hence skewing the sensitivity of the assay. Again, this emphasizes the need to include adequate controls for Ly specificity.

One of the best serological controls to include in experiments designed to test for specific effects of the Ly antisera was described by Shen et al. (39). This control includes the use of cells from relevant Ly congenic mice. For example, consider the hypothetical case of "Ly-X" where anti-Ly X.2 has been produced in B6 mice. If only cells from Ly X.2 animals are sensitive to cytotoxic treatment by anti-Ly X.2 but not cells from the congenic line B6-Ly X.1 then the cytotoxic effect is attributed to the Ly X system. Ideally, parallel results should be obtained using the reciprocal test with anti-Ly X.1 antibodies. This protocol, however, could not be always guaranteed to give satisfactory results. The authors attributed this failure to the then insufficient characterization and comprehension of the Ly system.

One of the most difficult problems that must be dealt with in the use of the Ly system is the one which serologists have been facing for many years. The problem is that the cytotoxic treatment of precursor cells with anti-Ly antisera, which subsequently leads to the loss of an immunological function, may not necessarily reflect the loss of the cell type which mediates that particular function. This becomes increasingly likely, especially in view of recent findings concerning the complexity of mutual cooperative interactions among lymphocytes in immune responses. Consider, for example, the assay for the generation of CTL. Cytotoxic treatment with the appropriate

Ly anti-sera was carried out on cells from normal spleen. After treatment, the cells were incubated with stimulator cells for 5 days. The results revealed the absence of CTL from cultures treated with anti-Ly 1 or anti-Ly 2. A mixture of cells from both treated populations did not reconstitute the cytotoxic response. Cells treated with NMS and complement responded positively. However, one cannot conclude from these results that the precursors of CTL are Ly 1^+, Ly $2,3^+$ (11,41). It is possible that the cytotoxic treatment was strongly effective in the elimination of an accessory cell, e.g. helper cell, the presence of which is required for the induction of CTL precursors. Helper cells of the type Ly 1^+, Ly $2,3^+$ have been described by Swain and Panfili (42). This indicates that the analysis of such data is extremely complex and the evidence is incomplete. More conclusive results, however, could be obtained if treatment was carried out on the effector level. At this stage the cells are fully differentiated and it seems likely that no further interactions are required. If killer effector cells were eliminated by either anti-Ly 1 or anti-Ly 2,3 antisera and a mixture of cells from both treated populations does not reconsitute the killer effect it is reasonable to conclude that the killer effector cells are Ly 1^+, Ly $2,3^+$. The probable advantages of a system which analyzes effector cells rather than precursor cells are further emphasized by the results included in this volume.

As can be seen from the discussion above, differences

based on the Ly phenotype of various categories of T cells could establish evidence for further subgroupings of functional T lymphocytes. The reality of this classification, depends of course, on the validity of the Ly typing system. It is not possible to provide definitive answers, but it seems likely that Ly classification will become increasingly important in the unravelling and understanding of the multiple and complex mechanisms of immunoregulation.

3. The major T cell subclasses

Growing evidence in the past decade has shown that there are three major subclasses of T cells: the helper cell, the cytotoxic cell, and the suppressor cell. Examination of the distribution of Ly antigens on T lymphocytes suggests that this functional heterogeneity is concordant with the diversity of expression of Ly surface markers. For example, the T helper cells are distinguished by the phenotype Ly 1^+, Ly $2,3^-$ and the suppressor cells are distinguished by the phenotype Ly 1^-, Ly $2,3^+$ (43). In these studies, the cytotoxic cells were characterized as Ly 1^-, Ly 23^+. Current information, however, suggests that these phenotypes are not characteristic for all mice. On superficial analysis, the emerging picture from these studies seems to be in a confused state. Nevertheless, a critical analysis of the available evidence suggests that each of these subclasses may be further divisible into

lesser subgroups. For simplicity of discussion, I shall consider each subclass separately.

a. Helper T cells

Cells of this subpopulation are capable of recognition of antigen and the delivery of helper activity to other functional cell types. They collaborate in the induction of an antibody response by B cells (2-4,43), the generation of cytotoxic T cells (10) and the development of delayed-type hypersensitivity (44). Ly typing showed that these cells possess the alloantigen phenotype Ly $1^+,2,3^-$ (43). A number of investigators, analyzing helper activity in a variety of experimental systems, have confirmed this characterization (45,46). However, while helper activity specific for a variety of antigens is well characterized, a number of complex questions as to possible subdivisions, the nature of H-2 reactivity (restriction) and the mechanism of action are still unresolved.

The ability of Ly 1 cells to provide helper activity has been demonstrated for the antibody response to a variety of antigens. Cantor and Boyse (43) showed that the removal of Ly 1^+ cell by treatment with anti-Ly 1 antisera and complement abolished the development of PFC activity to SRBC. Helper memory activity to KLH was also shown to reside almost entirely in the Ly 1 population of cells obtained from KLH-primed mice (46). Ly 1^+ cells were also found to be the effectors for helper function to streptococcal group A

vaccine (47). These authors concluded that Ly 1+ cells are not only the effectors of helper activity, but are already programmed to generate helper function prior to immunization. Whereas this claim might be correct for the helper effector function, these series of observations cannot adequately support the conclusion that helper precursor cells are also Ly 1+. Again, this is a case in point where, given the nature of the assay employed, it is difficult to distinguish between the precursor and the effector. The assay is a PFC assay in which the selected T cell population (i.e. T cells obtained after treatment with the appropriate anti-Ly antisera) are incubated with the test antigen (usually SRBC or a certain carrier) for 5 days. Under these circumstances it is possible that, as a result of the cytotoxic treatment with the antiserum, a fully differentiated helper or possibly an "amplifier" function has been eliminated and the helper precursors themselves have not been touched. The presence of "amplifier" cells, or helper cells which express the phenotype Ly 1+, Ly 2+, have been documented by Swain and Panfili (42) and Feldmann et al (45). In view of these studies, the analysis of Ly phenotype of the helper precursor cells becomes increasingly critical.

Current information suggests that Ly 1+ cells also contribute the major helper activity for the generation of CTL. Cantor and Boyse (43) showed that although the Ly 1+ cells do not themselves directly contribute to the cytotoxic effector cell population, they account for the major portion

of the proliferative reaction in mixed lymphocyte culture. These studies also showed that during the mixed lymphocyte culture cells from the Ly 1^+ subclass can enhance the generation of cytotoxic cells from prekiller cell population. Similarly, the results described in this volume (see below) demonstrate that the helper cells required for the generation of cytotoxic T cells from thymus responder cells are Ly 1^+.

A lymphocyte with a similar phenotype to the helper cell but functioning in a different experimental design is the cell responsible for the development of DTH. Huber et al. (44) provided evidence that participating cells in three different DTH systems, all involving SRBC as antigens, were found to be Ly 1^+, Ly $2,3^-$. Ramshaw et al (48) and Vadas et al. (49) confirmed these results and added that, unlike some helper cells, T cells responsible for DTH are Ia^-. The relevance of these data to the whole picture of immunoregulation will be expanded upon in later sections (see section 4).

The general contention from these studies seems to be that almost all investigators in this field agree that T helper cells possess a distinctive profile of Ly antigens which distinguishes them from most other subsets of T cells. This profile is characterized as Ly 1^+, Ly $2,3^-$. More recently, Swain and Panfili (42) demonstrated that while the allohelper cells induced by differences at the whole H-2 haplotype, I-region or Mls were Ly 1^+, the allohelper cells

induced by differences only at the H-2K and/or H-2D regions were Ly 1^+, Ly 2^+. Further evidence suggests that there may be helper cells which can only help a certain category of B cells of a particular allotype, or idiotype (46,50). If this is the case, further subdivision of this subclass of cells into subgroups seems possible.

b. Cytotoxic T cells

Much of our current understanding of the nature and mechanism of T cell-mediated cytotoxicity comes from *in vitro* studies using an assay first used extensively by Brunner *et al.* (51). Lytic activity is assayed by the ability of lymphoid cells to destroy target cells bearing surface antigens to which these lymphoid cells were originally sensitized. Usually the target cells are specific tumor cell lines and are labeled with ^{51}Cr isotope. The release of the ^{51}Cr into the supernate from target cells is indicative of the presence of cytotoxic cells in the effector population.

T cell-mediated cytotoxicity is apparently differs from lytic reactions brought about by antibody and complement. The former requires intimate contact between the effector cell and its homologous target, whereas the latter does not. The term cell-mediated cytoxocity was thus proposed (20).

Evidence to date suggests that there are three types of cell-mediated lysis: (1) antibody-dependent cell-mediated cytotoxicity (ADCC); (2) natural killers; (3) cytotoxicity

mediated by specifically sensitized T cells. This review will concentrate on the third type of cytotoxicity, i.e. killer cells generated in allogeneic systems. The former two types of cytotoxicity have been described elsewhere (20).

Specifically sensitized T cells can be generated in vitro or in vivo to allogeneic cells (8), Virus-infected cells (52), or cells chemically modified with TNP (41). The killing activity is specific in that the cytotoxic cells destroy target cells sharing antigenic determinants identical to, or cross-reacting with, the sensitizing antigen. The in vivo relevance of this form of cell-mediated cytotoxicity has long been suspected in the rejection of most grafts, the graft - versus - host reaction and possibly plays an important role in an immune defense mechanism against tumor cells (for references on this subject see references 20 and 53).

The most commonly used method for the generation of specifically allosensitized T cells is the one way mixed lymphocyte culture reaction. Responder cells are obtained from either spleen, lymph nodes or thymus and mixed with irradiated, or mitomycin C-treated MHC-disparate stimulator cells. This mixture is incubated for five days under optimal conditions (see Materials and Methods). Cells are then added to the appropriate ^{51}Cr-labeled target cells to measure the level of cytolytic activity.

Cytotoxic cells are sensitive to treatment with anti-theta serum and complement and are Ig^-. Early reports

indicate that cells from this subclass express the Ly phenotype Ly 1⁻, Ly 2,3⁺ (7,54-56). Recent evidence suggests that this Ly phenotype of the cytotoxic T cells is not characteristic of CTL from all strains of mice. Shiku et al. (54) demonstrated that while anti-Ly 1.2 antisera has no effect on CTL from C57Bl/6 mice (haplotype Ly 1.2, Ly 2.2) anti-Ly 1.1 antisera significantly reduced the lytic activity of cells from Bl/6 congenic mice (haplotype Ly 1.1, Ly 2.1). Both types of killer cells were susceptible to treatment with anti-Ly 2 antisera directed against the corresponding allele. Beverly et al. 1976 (55), using cells from CBA mice (haplotype H-2 Ly 1.1, Ly 2.1) as responders for the generation of CTL, found that the Ly phenotype of the cytotoxic T cells was Ly 1⁺, Ly 2,3⁺. Similar results were obtained in our system using the same strain of mice (see Results and Discussion below). These studies indicate that the strain of mice used in experiments designed to examine the distribution of Ly markers on the killer cells must be taken into consideration.

In an attempt to further characterize this subclass of T cells, Shiku et al. (57) demonstrated that when an isogenic tumor is the target of CTL (so that the effector is reacting against a non-MHC antigen) the Ly phenotype of the effectors is Ly 1⁺, Ly 2,3⁺. In the same study, the effector cells against an MHC allogeneic target were found to be Ly 1⁻, Ly 2,3⁺. Contrary results were found by Pang et al. (58) They immunized mice by infection with ectromelia virus.

Killer cells from these mice were treated with various anti Ly antisera and complement and then added to ectromelia-infected target cells to assay for cytotoxicity. They found that the predominant effector phenotype is Ly 1^-, Ly $2,3^+$, though the killer cells from the primary cytotoxic response showed some susceptibility to treatment with anti-Ly 1 antisera.

The evidence thus far is in favor of the presence of two subgroups of CTL. One group expresses the phenotype Ly 1^+, Ly $2,3^+$ and the other expresses the phenotype Ly 1^-, Ly $2,3^+$. While there is no known correlation between these two groups and a particular type of target cell, it seems likely that the Ly 1^-, Ly $2,3^+$ phenotype is characteristic of C57Bl/6 mice (H-2b) and the phenotype Ly 1^+, Ly $2,3^+$ is characteristic of CBA mice (H-2k). Can it be inferred from this that the CTL population from H-2b mice is always Ly 1^-, Ly $2,3^+$ and the CTL population from H-2k mice is always Ly 1^+, Ly $2,3^+$. Undoubtedly, this issue will become much clearer when results of genetic and biochemical analysis of the Ly system become available.

c. Suppressor T cells

After a decade of intensive research categorizing and characterizing the suppressor cell phenomenon, reasonable conclusions have been advanced. T suppressor cells can depress the development and the expression of both humoral and cell-mediated immune responses. In some instances the T

cell-mediated suppression is antigen-specific, i.e. only the response to the antigen against which the suppressor cells were activated is inhibited. In other instances suppression is antigen-nonspecific. That is, although the suppressor cells are activated by a specific antigen, the response to other test antigens are also suppressed. Suppressor T cells can also be induced by polyclonal activators such as concanavalin A. These suppressors can inhibit both cell-mediated and humoral immune responses, as well as proliferative responses to other mitogens. Comprehensive reviews on this subject have been published in a number of text books and scientific journals (59-62). The author, in his thesis on the antigen-specific suppression of T cells responses, has discussed a wide range of model systems in which suppressor cells were elicited and assessed in both humoral and cellular immunity (59). In this thesis I shall attempt to discuss new aspects of immunoregulation by examining the expression of the Ly surface markers on suppressor cells.

Most reports concerned with Ly-typing indicated that suppressor cells represent a heterogeneous subclass of T cells. Three subtypes of suppressor T cells have been identified, so far, each bearing a distinct Ly profile. Firstly, a subtype which exclusively expresses the phenotype Ly 1^-, Ly $2,3^+$ was characterized by Cantor et al. (7). This type of suppressor cell exerts a major antigen-specific suppressive function in the antibody response to SRBC (7)

and HRBC (48). These results were further extended to include suppressor cells involved in the regulation of the antibody response to KLH (55,63) and the production of allotype Iglb(46).

Secondly, a subtype of suppressor cells which was generated in mice undergoing graft-versus-host reaction has been characterized as Ly 1^+, Ly $2,3^+$ (64). These suppressor T cells were found to nonspecifically inhibit the in vitro antibody response to SRBC. In a slightly different system, nonspecific suppressor T cells which express the same Ly 1^+, Ly 2^+ phenotype were also identified by Mosier et al (65), Newborn mice infected at 18 hours of birth with mouse thymic virus develop, 10 to 16 days later, suppressor cells which inhibit antibody production by mature B cells. The ability of Ly 1^+, Ly $2,3^+$ T cells to exert a "feed back" inhibitory effect on the antibody response to SRBC was further demonstrated by Eardly et al (66). However, antigen specificity was not studied in this system. Suppressor cells of this phenotype can also suppress the induction of CTL (see results below). These suppressors are antigen specific, cortisone sensitive and radiation resistant T cells (67).

Thirdly, a subtype of suppressor cells has been characterized as Ly 1^+, Ly $2,3^-$. Ramshaw et al. showed that HRBC-specific T cells from mice expressing humoral immunity were able to suppress the induction of HRBC-specific DTH(48). Suppressor cells in this system were shown to be Ly 1^+, Ly $2,3^-$. A suppressor cell of this type, but acting in a

nonspecific fashion, was also identified by Watanabe et al. (68). SJL mice, "under appropriate conditions of immunization combined with irradiation", develop a high and persistent anti-DNP response. The transfer of spleen cells from untreated (normal) SJL mice selectively suppressed the IgE response *in vitro*. Elimination of Ly 1 cells from spleen cells before transfer abolished the suppressive effect, whereas removal of Ly 2 cells had no effect, indicating that the suppressor cells were Ly 1^+, Ly $2,3^-$. These suppressor cells show no antigen-specificity for carrier, or hapten.

Further evidence showing that Ly 1^+, Ly $2,3^-$, cells can have an inhibitory effect on the generation of antibody response was reported by Mcdougal et. al. (47). In this system antigen-pulsed macrophages were cultured with cortisone-resistant, nylon wool-purified, thymocytes for 4 days. Ly 1^+ cells from this culture were shown to specifically inhibit the PFC response of normal spleen cells. The authors indicated that the Ly 1^+ cells are probably not the effector suppressors. Instead, these cells could provide an ancillary cell that is required for the generation of suppressor cells from other T cell types. Their evidence does not, however, distinguish between the alternative phenotypes Ly 1^+, Ly $2,3^+$ and Ly 1^-, Ly $2,3^+$ for the suppressor effector cell.

While the net regulatory effect of all of the above described suppressor cells is inhibitory, their mechanism of action could be different. Herzenberg *et al.* (69) postulated

that the target of suppressors bearing Ly 1-, Ly 2,3+ markers is the Ly 1+ helper T cell. This conclusion was based on the observation that Ly 2,3+ suppressor cells from the Iglb allotype-suppressed mice exert a cytotoxic effect on the Ly 1+ helper T cells that are programmed to interact selectively with Iglb+ B lymphocytes. Further evidence suggesting that inhibitory T cells can inhibit the generation of helper T cells has been reported by Hamaoka et al. (70). T-T cell inhibitory interactions were also demonstrated in the induction of CTL. In this system several investigators have reported that the suppressor activity is directed against the responder cell population (71,72). This, of course, could be either the precursors for helper cell activity or the precursors for the generation of CTL.

Evidence for a T-B cell suppressor interaction which involved the suppressor cell acting directly on the B cell comes from the work of Basten et al. (73) who showed that the site of suppression in the inhibition of the anti-DNP.HGG response is the hapten-sensitive precursor cell (B cells). These findings do not, however indicate that the suppressor T cells are acting on the B cells alone. The possibility of the involvement of macrophages or helper cells in this inhibitory interaction cannot be ruled out.

The idea that negative regulatory effects may involve direct, or indirect, macrophage-T or macrophage-B cell interactions was verified by several investigators. Pierce et al (74) demonstrated that a Con-A-stimulated inhibitory

activity appeared to act on the splenic macrophages. Other evidence in favor of this comes from the work of Asherson and Zembala (75) and Basten et al. (73) on the cells mediating the suppressor effect. These studies demonstrated a requirement for adherent cells, probably macrophages. In this case, a macrophage could play either one of two roles. Firstly, a macrophage may receive an inhibitory signal and itself become inactivated. This would suggest that the macrophages are the final targets for the inhibitory activity, and a loss in their function leads to a loss of the immune response. Secondly, a macrophage may act as a mediator for suppressive activity and deliver the inhibitory signal to other responding parties, i.e. T cell precursors or B cell precursors. On an experimental basis, it is very difficult to distinguish which of these possibilities is taking place. Nevertheless, the concept that the actual mechanism of the regulatory events which take place in the immune response could involve the macrophages is an important one and demands further verification.

Although the use of the Ly antisera has been instrumental in the identification of the various categories of suppressor cells, difficult questions with regard to the mechanism of action and the physiological role of the suppressor cells remain unsolved. Most authors admit to the fact that, for something as complex as the inhibitory function of T cells in immune responses, multiple pathways of activation and interaction exist. Several of these

pathways are now being explored. The most important one, to my mind, is the study of the relationship between the inhibitory cells and other well characterized and better defined functions of T cells. I shall thus consider possible relationships between suppressor and helper T cells on the one hand, and between suppressor and cytotoxic T cells on the other.

4. The relationship between helper and suppressor T cells.

The interaction between an immunocompetent lymphocyte and the appropriate helper cell, in the presence of antigen, leads to induction. Conversely, the interaction between an immunocompetent lymphocyte and the appropriate suppressor cell, in the presence of antigen, leads to suppression. Although suppressor and helper cells have opposing functions, striking similarities exist between these two types of cells. Both cells bear theta antigen on their membranes and accordingly are thymus derived cells. By most reports both cells are antigen specific, though antigen-nonspecific suppression and help have been described. The precursors of helper and suppressor T cells are radiosensitive, whereas the helper and suppressor - effector functions are radio resistant (for references see section 3 parts a and c).

The antigen-specific suppressor and helper T cells may be considered to act via linked associative recognition (76-78). That is, both helper and suppressor regulatory

effects require recognition of at least two determinants on the same antigen. One determinant is recognized by a precursor cell, and the other is recognized by a regulatory cell (helper or suppressor) or its product. This apparent requirement for an antigen bridge allows regulatory signals to be delivered at short range, for example via cell to cell contact or by short-lived soluble factors, so that only precursor cells binding the appropriate antigen are either specifically induced or suppressed.

Antigen specific soluble mediators that affect both humoral and CMI responses have been obtained from both helper and suppressor T cells. Educated helper T cells elaborate a factor that replaces the requirements for helper T cells (79-81). Similarly, antigen-activated suppressor T cells can also elaborate biologically active and antigen - specific suppressor factors. Tada et al. (82) showed that the factor obtained by sonication of thymi and or spleens from KLH primed donors was capable of inhibiting primary and secondary IgG responses to DNP-KLH in vivo and in vitro. This factor was shown to be antigen specific and does not react with anti-Ig reagents. Further work indicated that this factor is at least partially a product of a gene located in a newly described I-subregion, termed IJ (83).

Suppressor and helper factors are remarkably similar in their physicochemical and immunochemical properties. Pierce and Kapp summarized these properties as follows (61): (1) Both helper and suppressor factors are antigen specific and

can be absorbed by immunoabsorbents of the inducing antigen. (2) The molecular weights of these factors are estimated to be in the range of 30,000 to 60,000 daltons. (3) Both factors show H-2 restriction and can be removed by alloantisera against products of the I-region of the H-2 complex. (4) Neither the helper nor the suppressor factor has any demonstratable reactivity to anti-Ig antibodies.

Although certain similarities between suppressor and helper T cells have been described, basic differences also exist between these two subpopulations of T cells. Helper T cells can be distinguished from suppressor cells on the basis of functional and physical grounds. Suppressor T cells are clearly different from helper T cells by virtue of the ultimate biological effect they exert. This fact does not a priori distinguish the cells mediating these opposite regulatory functions since, under variable conditions, the same cell could act differently. These conditions are manifest in the nature of antigen and the method of sensitization most apt to stimulate the development of suppressor cells or helper cells.

Examination of the expression of cell-surface diferentiation markers contributed additional evidence in support of the dissociation of suppressor T cells from helper T cells. As mentioned above helper cells express exclusively the phenotype Ly 1^+, Ly $2,3^-$. In contrast suppressor cells, depending on the assay system, exhibit any one of three Ly phenotypes: Ly 1^+, Ly $2,3^-$, Ly 1^-, Ly $2,3^+$

and Ly 1^+, Ly $2,3^+$.

The detection of inhibitory cells which bear the phenotype Ly 1^+, Ly $2,3^-$ indicate that at least this subtype of suppressor cells is not different from helper T cells. This has been interpreted to suggest that excess help could exert an inhibitory effect (84). Recently this concept has met a great deal of opposition especially after a number of observations on the physical and morphological properties of T cells have been described. The subject is currently in a rather confused state. The following discussion offers attractive possibilities in an attempt at resolving this conflict.

The concept that some suppressor effects could be due to "too much" help has been advanced in an hypothesis on immune class regulation postulated by Bretscher (85). Briefly, this theory states that different classes of precursor cells require different levels of helper cells for induction. Precursor cells for CMI require low levels of helper activity and precursor cells for humoral immunity require higher levels of helper activity. The theory also states that high levels of helper activity suppress the induction of CMI. The amount of helper activity induced is dependent on the concentration and the antigenicity (or "foreigness") of the antigen. The precursors for helper T cell effectors are induced at all but the most extreme doses of antigen. Low and high concentrations of antigen result in the generation of low helper activity and therefore only CMI

is induced. As the antigen concentration increases (i.e. at a medium dose of antigen), maximum helper activity is generated and humoral immunity is induced whereas CMI is suppressed. Similarly, an antigen with few foreign sites induces low levels of helper activity and only generates CMI, while an antigen with many foreign sites induces high levels of helper activity which results in the induction of humoral immunity and suppression of CMI. Since a shift to a high level of helper activity, sufficient to induce a humoral response, can suppress the induction of CMI, and since a physiologically effective immune response must have the potential to maintain stable CMI, a regulatory mechanism must be available for inhibiting the induction of helper activity. According to the theory (85), this mechanism could be mediated by a cell-mediated effector "antibody," or an antigen specific molecule co-ordinately expressed with CMI which acts on the helper cell precursors. This inhibition is referred to as repression, and is maintained as long as a cell-mediated immune response is required.

The above discussion on immunoregulation as proposed by Bretscher could be summerized as follows (Table I). Repressors are generated concomitantly with the induction of CMI, carry the Ly 2 marker, and can inhibit the development of humoral immunity by acting on the precursors of helper T cell effectors. Suppressors are induced with the humoral response, carry the Ly 1 marker, and inhibit the development of CMI by acting on the CMI precursors.

Table I. Inhibitory cells as described by Bretscher.

Type of inhibitor	Observed Ly marker	Class of immunity inhibited	Induced concomitantly with	Predicted mode of action
Repressors	Ly 2+	humoral	CMI	inhibits generation of help
Suppressors	Ly 1+	CMI	humoral	inhibits induction of CMI precursors

The finding by most workers that suppressor cells capable of suppressing antibody responses are Ly 1^-, Ly $2,3^+$ and that the helper cells are Ly 1^+, Ly $2,3^-$ is consistent with the above predictions of immune class regulation. That is, under conditions where humoral responses are being regulated, the suppressor T cells are different from the helper cells. The theory further postulates that too much help could suppress the induction of CMI, i.e. a rise in helper activity in an ongoing humoral response could inhbit CMI responses. Under these conditions, the theory predicts that helper cells are not different from suppressor cells. Support for this concept has been reported by Ramshaw et al who demonstrated that HRBC-specific T cells from mice expressing humoral immunity were able to suppress the induction of HRBC specific DTH (48). Suppressor cells in this system were shown to be Ly 1^+ cells. Since helper T cells are also Ly 1^+, the mechanism of this suppressive activity has been postulated to be due to the presence of high levels of specific helper T cells.

The question whether or not suppression of CMI responses could be due to "too much" help remains a matter of controversy. Much of this controversy stems from the fact that the nature and mechanism of the cellular interaction that takes place at the suppressor - target cell level has not been characterized. The theory does not rule out the possibility, however, that a series of interactions which could involve cell types other than the helper cells could

exist prior to the final step of the suppressor - target cell interaction. In the presence of a high level of helper cell activity inhibitory cells of the type Ly 1^+, Ly $2,3^+$ or Ly 1^- Ly $2,3^+$ could be induced. These inhibitory cells could in turn act to suppress the induction of CMI in two ways. Firstly, these cells could act on the helper cell precursor, preventing further differentiation to helper effector function and consequently inhibiting the induction of CMI. Consistent with this is the observation that the induction of CTL requires the presence of fully differentiated helper T cells (10). Secondly, the inhibitory cells could directly suppress the precursors of CMI from further differentiation into cell-mediated effector functions.

5. The relationship between cytotoxic T cells and suppressor cells

At present the evidence on the separation of suppressor cells from cytotoxic cells is less clear. The observation that, in some systems, suppressor cells and cytotoxic cells share the same Ly phenotype (Ly $1,^-$ Ly $2,3^+$) has lead to the inference that suppressor cells and cytotoxic cells belong to the same subclass of T cells (86). Further evidence in support of this hypothesis was reported by Fitch et al. (24). These workers observed that cells from five-day primary MLC, the optimum time for generating peak CTL, completely inhibited the generation of CTL *in vitro*. The inhibitory activity was antigen specific and was directed

toward the alloantigen for which the cells from primary MLC were cytotoxic. They showed that the presence of primary MLC cells having cytotoxic activity toward one alloantigen abolished the response to another non-cross-reacting alloantigen only when both antigens were present on the same F1 hybrid stimulating cells, whereas the response to the sensitizing alloantigen only, was inhibited in cultures where a mixture of cells expressing the two non-cross-reacting alloantigens were used as stimulator cells. In view of these observations, the authors concluded that the suppression of generation of CTL by irradiated cells from primary MLC involves inactivation of the allo-antigen-bearing stimulating cells as a result of residual cytotoxic activity of the primary MLC cells.

The foregoing hypothesis has much to commend it in that it assigns a role to suppressor cells consistent with a known T cell function, cytotoxicity. If suppressor cells were cytotoxic cells one could envisage a number of alternative modes by which suppression is achieved. Suppressor cells could specifically eliminate: (1) stimulator cells, i.e. the source of antigen, (2) antigen-reactive T helper or B cells; (3) antigen-bearing macrophages. In view of recent evidence, however, certain models of suppressor cell activity cannot be adequately explained via a cytotoxic mechanism. The data suggest, on the basis of several criteria such as size (87), cortisone sensitivity (26) and the elaboration of suppressor factor

(82) that suppressor T cells are functionally and physically distinct from cytotoxic T cells. These observations have been discussed elsewhere (59). Further evidence on the dissociation of suppressor T cells from cytotoxic T cells is considered later (see Chapter IV).

CHAPTER II

Materials and Methods

A. Materials

1. Animals

Male and female adults of the inbred strains BALB/c (H-2d), C3H-SwSn (H-2b), CBA/CaJ (H-2k) mice (5 to 12 weeks old) were used throughout.

2. Cell lines

P815 mastocytoma (H-2d, derived from DBA/2 mice) and EL4 leukemia (H-2b, derived from C57Bl/6 mice) were maintained *in vitro*. The P815 cells were grown in H-16 (Dulbecco's modified) + 10% fetal calf serum culture medium and the EL4 cells were grown in F-15 (Eagle's minimal essential) culture medium + 10% fetal calf serum. Cells were transferred every two days into tissue culture flasks (Corning 75 cm^2/tissue culture flask) and grown at 37°C in 10% CO2 air atmosphere. At the time of transfer, P815 cells were set at $5x10^4$ cells/cc and EL 4 cells were set at $1x10^5$ cells/cc. As a safeguard, gentamicin was included at 50-75 ug/ml final concentration. Both cells lines were a gift from Dr. E. Sabbadini, University of Manitoba, Canada.

3. Tissue culture medium

Eagles' Minimal Essential (F-15), Dulbecco's Modified (H-16) and Leibovitz media were obtained in powder form from Grand Island Biological Company (Gibco). All cultures were grown in F-15 supplemented with 10% F.C.S. (Gibco) and 50 ug/ml gentamicin, Microbiological Associates. F-15 contained a final concentration of 10^{-4}M mercaptoethanol, 20 ug/ml penicillin streptomycin (Gibco) and 0.8 mM of L. Glutamine (Gibco).

4. Tissue culture vessels

a. Microtiter trays: v-bottom trays, 96 wells/tray (Cooke Laboratory Products) were obtained from Microbiological Associates, Bethesda, Maryland.

b. Polyacrylamide rafts: Marbrook polyacrylamide tissue culture vessels (rafts) were prepared according to the method described by Marbrook and Haskill (88) and Pilarski and Borshevsky (89). The materials and steps involved in making these rafts are as follows:

1) Acrylamide (Eastern Kodak Co.) Fisher Scientific.

2) N, N' Methylene Bisacrylamide H.P. (Ames Company, International Scientific.

3) Ammonium persulfate (Baker Chemical Co.), Canlab.

4) N, N, N', N'-Tetramethylethylene Diamine, 99%, TEMED (Aldrich Chemical Co.).

5) Molds and glass covers: The molds are designed in such a way that the bottom half of the inner chamber of the raft is subdivided into 36 individual V-bottom wells. This allows a continuous flow of medium over all the 36 wells in a raft. Each raft holds a total volume of one ml. There are nine chambers per mold and each chamber holds one raft. These molds are constructed from two layers of lucite.

Dimensions in cm:

	Length	x	Width	x	Depth
Molds	14.5	x	14.5	x	1.1
Chambers	3.4	x	3.4	x	0.6
Glass covers	20	x	20	x	0.3

5. Antisera

Alloantisera directed against the Ly, Ia and IJ antigens were prepared by Dr. I.F.C. McKenzie, tested for specificity and cytotoxicity on the appropriate strains of mice and shipped frozen in dry ice. These antisera were used at a concentration giving optimum lysis, e.g. 1/5, 1/10 and 1/20. A summary on the information about these antisera is shown in Table II.

6. Source and dose of irradiation

Cells were give 1,500 rads of -irradiation from an Atomic Energy of Canada Limited gamma cell 40 which contained cesium 137. The source operates at a rate of 99.89 rads/min.

Table II. Summary of the codes, titer and method of preparation of the antisera utilized in this study.

# of Antiserum	Specificity	Donor	Recipient	Titer	Target
First Batch					
458	Ly 1.1	B6.Ly-1a	(B10.AKM x 129) F1	>1/256 1/64	Thymus LN
678	Ly 2.1	CE	C57BR/cd	>1/256 1/32	Thymus LN
754	Ly 7.2	CXBK	(B6.C-H-2d x CXBG) F1	1/64	Spleen
701	Iak	A.TL	(A.TH x B10.S) F1	>1/2000	Spleen
Second Batch					
748	Iak	A.TL	A.TH	>1/2000	Spleen
766	IJk	B10.S(9R)	(B10.HTT x BALB/c) F1	1/128	Spleen
466	Ly-1.1	B6-Ly-1a	(129 x B10.C-H-3c) F1	>1/256	Thymus
744	Ly-2.1	CE	B10.BR	1/64	Thymus
918	Ly-5.1	A.SW	(DA x SJL) F1	1/128	Thymus
922	Ly-6.1	C3H	(C3H.B6 x B6) F1	1/32	Spleen

B. Methods

The steps involved in making the rafts are described below:

1. Preparation of ammonium persulfate solution: 5.25 gm were dissolved in a liter of double distilled water. The solution does not keep well for longer than one month. If this volume is too much to be used in a period of one month, smaller amounts should be prepared. This solution was stored at 4°C at all times.
2. Preparation of acrylamide solution:
   a. 157.5 gm of acrylamide and 3.75 gm of Bisacrylamide were dissolved in one liter of double distilled water in a glass beaker. Using a stirring bar the solution was allowed to mix on a magnetic stirrer for 10-15 mins.
   b. Using Whatman H4 filter paper, the solution was filtered into glass bottles and the bottles were wrapped with aluminum foil.
   c. The bottles were stored in a dark place at room temperature.

   Caution: Acrylamide is a neurotoxin so the use of gloves when preparing these solutions, and a face mask when weighing out the acrylamide is a manditory procedure.
3. Preparation of polyacrylamide gel rafts:

a. In a glass beaker, the following materials were mixed as indicated and in the following order:

144 ml acrylamide solution
36 ml ammonium persulfate
93 ml double distilled water
0.15 ml TEMED

b. The mixture was stirred and quickly poured to fill each chamber of the mold right to the top. With a batch of this size, one should be able to fill 4 molds.

c. A glass cover was placed over each mold by sliding the glass cover, starting from one edge, across the mold while maintaining a slight pressure in order to keep the glass cover and the mold in air tight contact. In this process one should try to avoid making air bubbles. The molds then are let to stand for 20 min.

d. Each mold was separated from the glass cover by inserting a spatula between them and exerting gentle pressure. The rafts should appear on the surface of the glass cover. Using a wet spatula, the rafts were removed and collected in a beaker. Autoclavable nalgene plastic containers with lids are ideal for this purpose. After collecting the rafts from all the molds, the beaker was filled with saline and stored at 4°C.

e. The rafts received 4 changes of saline at 12-24 hr.

intervals.

f. After the fourth change of saline, the rafts were autoclaved in saline.

g. Two days prior to their use in culture, the rafts were equilibrated with 2 changes of medium (F-15 without fetal calf serum).

h. These rafts float in 15x60 mm petri dish containing 4 ml of tissue culture medium. One ml of the appropriate cell suspension is pipetted into each raft.

4. In vitro generation of cytotoxic T lymphocytes (CTL)

This system utilizes the one way mixed lymphocyte culture method. The method involves incubating a given number of cells, which are referred to as responders, with another given number of cells, which are referred to as stimulators. Stimulator cells are either pretreated with mitomycin or irradiated with a lethal dose of irradiation. Cells treated in this manner are limited in their capacity to divide and differentiate. Usually, responders and stimulators are chosen from histoincompatible strains of mice. After a given period of incubation, cultures are assayed for a particular response; for example, thymidine incorporation or generation of CTL.

The steps involved in the generation of CTL are described below:

a. Preparation of stimulator cells: Using sterile technique, spleens are removed from the animals and

minced in Leibovitz medium and 10% F.C.S. Stimulator cells are prepared according to the method of Lafferty et al. (90) in which stimulator spleen cells are preincubated fo 90 mins, in a 37°C water bath. Immediately before culture, cells are irradiated, washed and resuspended in tissue culture medium.

b. Preparation of responder cells: Responder cells are prepared from either spleens or mesenteric lymph nodes. Using sterile technique, the proper tissue is minced in Leibovitz and 10% FCS. Cells are carefully washed and resuspended in the proper volume of tissue culture medium.

Viability counts are determined using dye exclusion method.

c. Setting up the cultures:

1. In rafts: The appropriate number of responder and stimulator cells are mixed together and one ml of this cell mixture is pipetted into each raft. The usual protocol is to use of $5\text{-}50\times10^5$ responder cells and $8\text{-}16\times10^6$ stimulator cells per raft.
2. In microtiter trays: These generally contain $1\text{-}10\times10^5$ responders and 10×10^5 stimulators prepared as described above. At least four replicate cultures (wells) are set up per group. Cultures are incubated at 37°C in 10% CO2 air atmosphere. After a given period of incubation, cultures were assayed for the

presence of CTL. Optimum levels of cytotoxicity are consistently observed after five days of incubation.

d. Labelling of target cells: The required number of cells from the appropriate tumor cells line were centrifuged and resuspended in the proper medium at a concentration of $5x10^6$ cells/ml. Radioactive $Na^{51}CrO4$ was added to a final concentration of 100 uCi/ml and the cells were allowed to incorporate label for 60 mins. at 37°C. They were then washed in F15 and 10% FCS 4 times and resuspended in tissue culture medium at a concentration of $1x10^6$/ml. These cells were then added to the cytotoxic lymphocytes at 0.1 ml/well ($1x10^5$ targets).

e. Assay of cultures from rafts: Rafts are harvested by resuspending each of the 36 pellets/raft into a common pool and pipetting the cell mixture into a centrifuge tube. Cells are then centrifuged and resuspended in 1 ml/raft of fresh culture medium. In order to accurately determine the cytotoxicity of the whole population of cells, several dilutions of those cells are always assayed. In general, the cells are assayed at 1/10 of a culture/assay well, 1/30 of a culture/assay well, and 1/60 of a culture/assay well. Each was assayed in triplicate. This range insures that at least one of the dilutions will fall in the linear portion of the % lysis vs cell dose curve where the amount of killing is directly proportional to the number of lymphocytes added. The cytotoxicity assay is done in microtiter

trays. Each assay well contains 0.1 ml (10^5) ^{51}Cr labeled target cells and 0.1 ml of cells from each dilution of cultured lymphocytes. Microtiter trays with V-shaped wells allow maximal target cell lysis. The assays are incubated at 37°C in 10% CO2 air for four hours. The top 0.1 ml is removed and counted for supernatant ^{51}Cr cpm.

f. Assay of cultures in microtiter trays: The medium in each well is removed by suction and the cells are resuspended in 0.1 ml of fresh culture meduim per well. To each well, 0.1 ml of ^{51}Cr-labelled target cells ($1x10^5$ targets) is added.

In order to determine the total releasable ^{51}Cr, 0.1 ml of 0.05% Triton-X-100 detergent is added to 0.1 ml (10^5 cells) ^{51}Cr-labelled targets. Spontaneous lysis is determined by incubating 0.1 ml of targets with 0.1 ml of medium. These are done in six replicate wells. All assays are incubated at 37° in 10% CO2-air mixture for 4-6 hours. The top 0.1 ml of supernatant from each well is then collected in a glass tube and counted in a gamma counter. Per cent specific lysis is calculated as follows:

$$\% \text{ specific lysis} = \frac{\text{sample cmp-spontaneous cpm}}{\text{detergent lysis cpm}} \times 100$$

5. The in vitro generation of suppressor and helper cells

Suppressor and helper cells are generated in a mixed lymphocyte culture in which $3\text{-}5x10^6$ responder cells and

$5\text{-}8 \times 10^6$ irradiated stimulator cells are co-cultured in Marbrook acrylamide rafts. Usually, the responder cells are prepared from adult CBA spleens and the stimulator cells are prepared from BALB/c spleens. Three days later, the cells from these first step cultures are washed, irradiated, and divided intc two parts: one part is assayed for suppressive activity and the second is assayed for helper activity. In order to assay for help or suppression, cells from first step cultures were added to a fresh MLC, referred to as the second step culture. The preparation of second step cultures for each assay is described below.

6. Suppressor cell assay

Suppressor cell activity was determined by adding irradiated first step cells to second step culture. Responder cells for the second step cultures were obtained from either CBA/CaJ adult spleen or 5-week-old thymus. Spleen cells were used at $1\text{-}5 \times 10^5$ cell/culture and thymus cells were used at 10^6 cells/culture. These cultures were stimulated by 3×10^5 irradiated BALB/c spleen cells. These cell numbers were found optimum for the generation of a positive cytotoxic response. To this mixture, graded numbers of irradiated cells from first step cultures were added to assess inhibitory activity. Second step cultures were prepared in V-bottom 96 well microtiter plates (Cooke Laboratories). Each experimental group included four to eight replicate cultures. After five days of incubation at

37° and in 10% CO2 air atmosphere, cultures were assayed for cytotoxic activity as described above.

7. Helper cell assay

For this assay, second step cultures were prepared from the same pool of 5-week-old CBA/CaJ thymus cells used in the suppressor assay. The number of responder cells in this assay was 1-3x10^5 cells. These cultures consistently provide a negative, or weak, cytotoxic response which is essential for the evaluation of the helper effect on the generation of CTL. To this mixture, graded numbers of cells from the same pool of first step cultures used in the suppressor assay were added to assess helper activity. These second step cultures were also set up in V bottom microtiter trays; each group consisting of 4-8 replicate culture wells. After five days of incubation at 37°C and in 10% CO2 air atmosphere, cultures were assayed for cytotoxic activity as described above.

8. Complement (C)-dependent cytotoxicity

Cells at 10^7 - 2x10^7/ml were incubated with the appropriate antisera in polystyrene culture tubes (Corning, 15 ml) for 30 min. in a 37° water bath. Cells were spun down (1000 rpm) and absorbed rabbit complement was added at a final dilution of 1/5-1/8. After incubation in a 37°C water bath, for 45 min., cells were washed once and the viable count was determined by an eosin dye exclusion method. Cells

were also treated with normal mouse serum (NMS) as a control for nonspecific effects of the treatment. After treatment cells were resuspended in volumes equal to that of the NMS control and used as such. No enrichment was made for any group of cells after treatment. Whenever possible, the experiments were designed in such a way that the anti-Ly sera were used as controls for each other.

CHAPTER III

Surface markers on the T cells that regulate Cytotoxic T cells responses

A. Introduction

Thymus-derived lymphocytes (T cells) participate in a complex netwcrk of immunoregulatory interactions which are both positive (helper effects) and negative (suppressive effects) in character. There is increasing evidence that each of these two functions is mediated by distinct subclasses of T cells (38,43-49). These subclasses were defined by utilizing alloantisera raised against a panel of T lymphocyte differentiation antigens (Ly antigens), originally developed by Boyse and colleagues (32). This work revealed that T helper cells bear Ly-1 but not Ly-2 antigen ($Ly\text{-}1^+$) and suppressor T cells bear Ly-2 but not Ly-1 antigen ($Ly\text{-}2^+$). A third subclass of T cells which bear both Ly 1 and Ly 2 ($Ly\ 1^+2^+$) antigens has also been described. The differentiation pathways and the biological properties of these subclasses of T cells have been reviewed above (See Chapter 1).

The regulatory interactions of T helper and T suppressor cells have been extensively studied, mostly in terms of their ability to regulate antibody responses to a variety of antigens. The T-T cell interactions which occur in cell-mediated immune responses are less well

characterized. An *in vitro* system in which helper T cells and suppressor T cells were generated and assayed for their ability to regulate the cytotoxic T cell response to alloantigens has been developed (12,67). The most important advantage of this system is that it allows critical evaluation of both the relationship between helper T cells and suppressor T cells and the relationship between suppressor T cells and cytotoxic T cells in one experiment. In the work to be described (Chapter III, section B) the distribution of Ly and I-region antigens on the helper and suppressor effector cells have been examined. The surface phenotype of the cytotoxic T cells generated in this system is also discussed. The results show that the allo-antigen specific effector helper cells, suppressor cells and killer cells all represent physically distinct T cell subclasses. Furthermore, the Ly phenotype of the suppressor cell changes as a function of the time of incubation of the culture in which it is generated.

B. Results

1. Is suppression "too much" help?

The theory on immune class regulation postulates that there is an inverse relationship between the level of help and the antigen dose (84). That is, in the presence of low dose of antigen a high level of helper activity is required to trigger the response whereas in the presence of relatively high dose of antigen a low level of helper activity is sufficient to trigger the response. In order to examine whether or not such a relationship exists in the regulation of CTL an experiment was designed where the effect of varying the number of stimulator cells on the activity of inhibitory cells was tested. CBA anti-BALB/c cells, harvested on day 3 from a mixed lymphocyte culture (first step cells) were washed, irradiated and added, in the numbers indicated (Table III), to a second MLC to assay for their suppressive ability in the presence of varying numbers of stimulator cells. The second MLC was prepared with CBA spleen responder cells and irradiated BALB/c stimulator cells. The number of stimulator cells included a comprehensive range from $3x10^4$ - to $5x10^6$ cells. The results from two independent experiments are shown in Table III. One experiment was prepared using $1x10^5$ responder cells and the other was prepared using $3x10^5$ responder cells. Peak cytotoxicity was observed at a medium dose of antigen (lines

Table III. The relationship between the number of stimulator cells and the effect of first-step cells in second step cultures a).

Lines	Stimulator cells x10^{-5}	First-step cells x10^{-5}	% Specific lysis b) Number of responder cells 1x10^5	3x10^5
1	50	-	44.5±4.0	49.4±3.6
2		3	3.9±0.1	3.4±0.4
3		1	6.8±0.7	14.4±1.6
4		0.3	11.6±1.6	38.8±3.2
5	3	-	83.1±2.7	76.2±1.7
6		3	4.9±0.1	14.3±2.7
7		1	11.6±1.4	29.2±5.8
8		0.3	29.4±3.6	43.9±7.8
9	1	-	74.7±4.3	88.0±1.6
10		3	5.7±1.6	22.4±3.4
11		1	21.6±3.5	44.4±6.4
12		0.3	28.3±2.0	45.6±3.4
13	0.3	-	5.1±0.6	17.4±2.4
14		3	3.1±0.2	18.0±2.7
15		1	24.7±1.6	44.9±5.8
16		0.3	28.3±4.1	41.6±4.1
17	3 c)	3	3.4±0.9	

Table III cont'd.

a) Irradiated first-step cells (CBA anti-BALB/c) were added to second step culture prepared with the indicated number of CBA spleen responder cells and varying numbers of irradiated BALB/c spleen stimulator cells. Cells were cultured in microtiter trays for 5 days.

b) Mean values of 6 replicate cultures ±S.E.; spontaneous ^{51}Cr release 613±61; detergent ^{51}Cr releast 3540±248.

c) No responder cells added, irradiated first-step cells were cultured with stimulator cells only.

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5 and 9). In the presence of extreme doses of antigen the cytotoxic response varied significantly. At high dose of antigen the cytotoxic response is positive but still below optimum (line 1) whereas in the presence of low antigen dose the response is either negative or relatively weak (line 13). Under these conditions the regulatory effect of cells from first step cultures was most intriguing. For simplicity we chose to compare the results obtained from groups using $3x10^5$ and $3x10^4$ stimulator cells. The cytotoxic response of second step cultures stimulated by $3x10^5$ cells was completely suppressed in the presence of $1x10^5$ cells from first step cultures, although the suppressive effect was less dramatic in the experiment where $3x10^5$ responder cells were used in second step cultures. In contrast, cultures stimulated by $3x10^4$ cells (low antigen dose) responded

positively in the presence of the same number of first step cells ($1x10^5$). Under these conditions the regulatory effect of cells from first step cultures shifts from suppression to help. This observation is consistent with the concept that "too much" help can suppress the induction of CMI.

2. Is suppression due to elimination of antigen?

The above observation is also consistent with the contention that suppressor cells are not cytotoxic cells. If suppression was due to elimination of stimulator cells (antigen) as a result of anti-stimulator cell cytotoxicity, one would expect that suppression should be more efficient in the presence of relatively low numbers of stimulator cells. In other words, as the number of stimulator cells in the second culture decreases, this hypothesis predicts that fewer cells from first step cultures should be required to suppress the response. In fact, the opposite of this prediction is true. We observed that, in the presence of $3x10^5$ stimulator cells, the cytotoxic response is strongly positive Table III (83% lysis, line 5). A tenfold reduction of the number of stimulator cells ($3x10^4$) gives either no response or a very weak response (5% or 17% lysis Table III, line 13). Addition of $1x10^5$ cells from first step cultures suppressed the response in the presence of a high dose of antigen ($5x10^6$, $3x10^5$, or $1x10^5$ stimulator cells). In contrast to this, the same number of first step cells ($1x10^5$) actually helps the response in the presence of a low

dose of antigen; 24 or 45% lysis by cells from culture containing $3x10^4$ stimulator cells (Table III, line 15). This experiment included a comprehensive range of cell numbers from first step cultures. The effect of first step cells was dependent upon the number of stimulator cells in the second culture at all cell numbers tested. These effects were also consistently observed at two different levels of CBA responder cells (Table III, columns 3 and 4). These observations thus indicate that elimination of antigen by cytotoxic cells present in first step cultures cannot be the mechanism by which the suppressive effect is achieved.

3. A comparison of the suppressive effect of cells from first-step cultures on responder cells from adult spleen and responder cells from 5-week-old thymus

T cell-mediated suppression of the induction of CTL was previously assayed in a system where spleen cells from adult CBA mice were used as responder cells in second-step cultures (67). We extended this assay to a system where thymocytes are used as responder cells. Responder cells for second step culture were prepared from either adult CBA spleen or 5-week-old CBA thymus. The number of irradiated stimulator cells remained constant for all second-step cultures. Graded numbers of irradiated cells from first-step cultures were added to these cultures which, after 5 days of incubation, were assayed for cytotoxicity. The data from a representative experiment is shown in Table IV. Cultures

Table IV. A comparison of the suppressive effect by first-step cells on spleen responder cells and thymus responder cells.

Responder cells	Cells from 3-day first-step cultures	% specific lysis
$3x10^5$ Thymus	-	7 ± 3
	$3x10^4$	0
	$1x10^4$	26 ± 3
$1x10^6$ Thymus	-	45 ± 5
	$3x10^4$	0
	$1x10^4$	9 ± 5
$3x10^5$ Spleen	-	64 ± 2
	$3x10^4$	16 ± 4
	$1x10^4$	35 ± 3

Responder cells for second-step cultures were prepared from either adult CBA spleen or 5-week-old CBA thymus. Cultures were stimulated with $3x10^5$ cells from BALB/c spleen and the indicated numbers of first-step cells from 3-day cultures were added. After 5 days of incubation, second-step cultures were assayed for cytotoxicity. Values represent the arithmetic mean ± S.E. of six replicate cultures. This observation was repeated three times and the results were consistent.

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containing 10^6 responder thymocytes generated a positive cytotoxic response. The response was suppressed by the presence of inhibitory cells from 3-day first-step cultures. This suppressive effect was comparable to that observed in cultures where spleen cells were used as responder cells. It was found that cells from first-step culture have the same suppressive effect on the generation of cytotoxicity from second step cultures prepared with either adult spleen or with 10^6 5-week-old thymus cells. In contrast, cultures prepared with a low number ($3x10^5$) of thymus responder cells generated a minimal cytotoxic response. A positive response is obtained following the addition of cells from 3-day first-step cultures. This observation is characteristic of the helper assay described previously by Baum and Pilarski (12). They demonstrated that, under conditions where low numbers ($1-5x10^5$) of cells from 5-week-old thymus were used as responder cells in second-step cultures, helper cells are required for the generation of cytotoxicity. These observations indicate that the ability of irradiated T cells from 3-day first-step cultures to exert a helper or a suppressive effect on the generation of cytotoxicity depends on the conditions employed in the assay system. For example, low numbers of thymus cells from 5-week-old thymus, which alone are unable to generate a strong cytotoxic response, can produce cytotoxic T cells in the presence of low numbers of helper cells from 3-day first-step cultures. On the other hand, a 3-fold, or more, increase in the number of thymus

cells from the same mice will generate a positive cytotoxic response. In this case, the addition of graded numbers of cells from the same 3-day first-step cultures inhibits the response.

These observations were utilized in a system suitable for assaying for helper activity, suppression and cytotoxicity in one experiment. The major advantage of this system is that it allows critical characterization of the nature of the regulatory interactions in cytotoxic responses.

4. Ly surface markers of inhibitory cells obtained from first-step cultures

Inhibitory cells obtained from 3-day cultures of CBA spleen cells stimulated by BALB/c spleen cells were treated with various anti-Ly antisera and complement. They were then irradiated and assayed for their ability to suppress the induction of CTL in a second MLC. We found that the inhibitory activity was reduced at least 3-fold after treatment with anti-Ly 2.1 (Fig. 1). Similarly, treatment with anti-Ly 1.1 also abrogated the inhibitory effect by approximately 3-fold. This indicates that the inhibition of the generation of CTL is mediated by either a single subset of cells which express both the Ly 1.1 and the Ly 2.1 markers on their surface or by two subsets of cells, one of which expresses the Ly 1.1 marker while the other expresses the Ly 2.1 marker. In order to find out which one of these

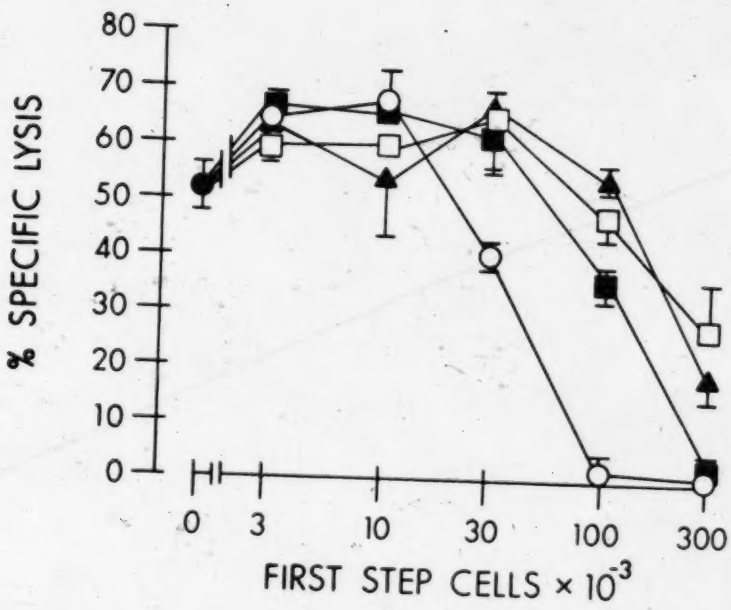

Figure 1. Ly Markers on inhibitory cells. Cells were obtained from 3-day first-step cultures of CBA spleen cells stimulated by BALB/c irradiated spleen cells and treated with NMS, anti-Ly 1, and anti-Ly 2 antisera. Then they were added to second-step cultures to assay for their ability to suppress the induction of CTL. Second-step cultures were prepared by mixing $1x10^6$ thymus cells from 5-week-old CBA mice and irradiated BALB/c spleen cells. Viable counts after treatment were as follows: NMS, $7x10^6$; anti-Ly 1, $3x10^6$; anti-Ly 2, $3x10^6$. Results are expressed in the arithmetic mean - S.E. of 4 replicate cultures.

Figure 1 cont'd.

• = no first step cells added

O = NMS

◓ = anti-Ly 1.1

◻ = anti-Ly 2.1

▪ = Mixture of anti-Ly 1.1 and anti-Ly 2.1 separately-treated populations.

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possibilities is correct, a mixture of cells from anti-Ly 1.1 and anti-Ly 2.1-treated populations was assayed for inhibitory activity. The results indicate that $1x10^5$ cells from this mixture have no inhibitory effect, although an equivalent number of the NMS-treated control cells gave 100% suppression. This indicates that the inhibitory cell population present in three-day first-step cells at high frequency bears both Ly 1.1 and Ly 2.1 antigens. In a second experiment of this type, an inhibitory cell bearing both Ly 1.1 and Ly 2.1 markers was also observed (Fig. 2, $3x10^4$ first step cells added). However, the results obtained for a higher number of first step cells ($1x10^5$) indicate a second, less frequent, population of inhibitory cells. At this number of first step cells, the inhibitory cells are sensitive to treatment with anti-Ly 2.1 but not to anti-Ly 1.1. In addition, the same cell number from the mixture control exhibited a strong inhibitory effect. This suggests

that a cell which bears Ly 2.1 but not Ly 1.1 is also involved in mediating an inhibitory effect on the generation of CTL, but this cell is present in three-fold lower numbers in three-day first-step cells than is the inhibitory cell which bears both Ly 1.1 and Ly 2.1 markers.

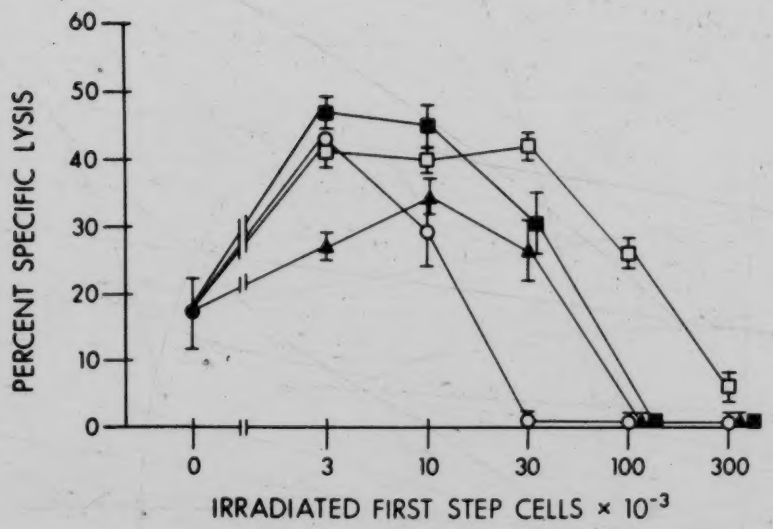

Figure 2. Ly phenotype of suppressor cells. First-step cells from the same cultures assayed for help in the experiment of Figure 4 were assayed for inhibitory activity. Various doses of first-step cells were added to second-step cultures containing 10×10^5 CBA thymus cells responding to 3×10^5 irradiated BALB spleen cells. This pattern of results has been observed in several comparable experiments. Viable cell recovery was: NMS 3.4×10^6/ml, anti-Ly 1.1 1.1×10^6/ml, and anti-Ly 2.1 1.8×10^6/ml.

● = no first-step cells

○ = NMS-treated first-step cells

▲ = anti-Ly 1.1-treated first-step cells

□ = anti-Ly 2.1-treated first-step cells

■ = mixture of anti-Ly 1.1 and 2.1-treated cells

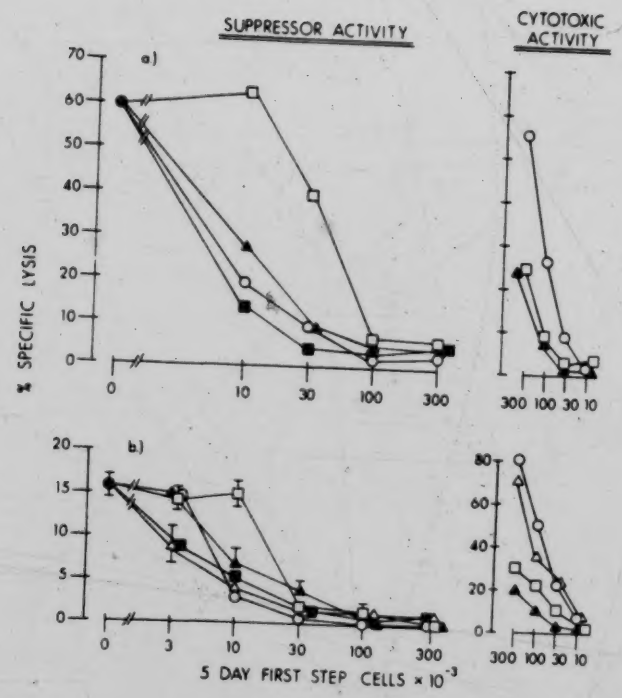

Figure 3. Suppressor cell phenotype at day-5 of culture. First-step cells were harvested from 5-day cultures, treated with the appropriate sera and assayed for suppressor activity as detailed in Figure 2. Parts (a) and (b) represent two independently performed experiments. Viable cell x10^{-6} recovery was: NMS a)=6, b)=8; anti-Ly 1.1 a)=3, b)=4; anti-Ly 2.1 a) 2, b)=5; anti-IJ=8.
• = no first-step cells, O = NMS-treated, ▲ = anti-Ly 1.1-treated cells, □ = anti-Ly 2.1-treated cells, ■ = mixture of anti-Ly 1.1 anti-Ly 2.1-treated, = anti-IJk-treated

Table V. Antigen specificity of 5 day suppressor cells.

Splenic Responder Cells $x10^{-5}$	Type of Stimulator cells	% specific lysis # of first step cells added				
		None	$3x10^5$	$1x10^5$	$3x10^4$	$1x10^4$
$1x10^5$	BALB/c	34±7	2±0	5±3	16±7	14±6
$5x10^5$	BALC/c	33±5	6±1	14±1	27±9	26±6
$1x10^5$	C3H.SW/Sn	21±6	22±9	26±8	39±12	31±3
$5x10^5$	C3H.SW/Sn	38±8	39±15	34±5	51±7	41±3

The antigen specificity of the suppressor activity of cells from a CBA anti-BALB/c five day first step culture was assayed using second step cultures prepared with CBA splenic responder cells, in the numbers indicated, and two types of stimulator cells: BALB/c (H-2d) irradiated spleen cells, the antigen against C3H.Sw/Sn (H-2b). Both stimulator cells were used at $3x10^5$ cells/culture. Values represent the mean ± S.D. of 4 replicate cultures.

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The presence of two different subsets of inhibitory cells in 3-day first-step culture prompted an investigation to determine whether or not at a later period of incubaton (for example, 5 days), the Ly phenotype of the inhibitory cells remained mainly Ly 1^+, Ly 2^+ or shifted to an Ly 1^-, Ly 2^+ phenotype. Cells from CBA anti-BALB first-step cultures were harvested at day 5. They were treated with the appropriate sera and assayed for suppressor activity in a second-step culture. The results from a representative

experiment are shown in Fig. 3. Treatment with anti-Ly 2.1 eliminated the majority of the suppressor activity (3-10 fold) whereas treatment with anti-Ly 1.1 had no effect. A mixture of anti-Ly 1 and anti-Ly 2-treated cells exerted a strong inhibitory effect comparable to that of the NMS-treated controls. These data demonstrate that inhibitory cells from 5-day cultures bear the Ly 2.1 marker but do not bear the Ly 1.1 marker.

Results from subsequent experiments showed that, like the 3-day suppressors, the 5-day suppressor cells are antigen-specific (Table V).

5. Helper cells

The effects of treatment with various Ly sera on the activity of helper cells was also tested. Using a helper cell assay, aliquots from the same CBA anti-BALB/c first step cells used in Figure 2 were treated with various anti-Ly sera, irradiated and added to a second step culture to test for their helper activity. As shown in Figure 4, the helper activity observed at the lowest cell number tested ($3x10^3$) is virtually removed from the anti-Ly 1.1-treated population. In contrast, treatment with anti-Ly 2.1 has no effect. At higher cell numbers, $1x10^4$ and $3x10^4$, a significant helper effect is still observed after treatment with anti-Ly 1.1, indicating that helper activity is reduced but not eliminated by treatment. This helper effect is, however, lower than and significantly different from that of

the anti-Ly 2.1-treated population, especially at the cell dose which gives optimal helper activity ($1x10^4$). The helper effect from a mixture of cells from anti-Ly 1.1 and anti-Ly 2.1-treated lots is parallel to the helper effect obtained from anti-Ly 2.1-treated cells. This indicates that the reduction of helper activity seen in anti-Ly 1.1-treated populations is due to the elimination of Ly 1.1-bearing helper cells, not to the presence of an inhibitory activity predominant in anti-Ly 1.1-treated cells.

Further examination of the nature of cytotoxic response in this curve (Fig. 4) confirms the presence of the inhibitory cell which bears Ly 1.1 and Ly 2.1 markers (see Figures 1 and 2). This is evident from the following observation. In the presence of $10\text{-}30x10^3$ cell from NMS-treated first step cultures the cytotoxic response is weak or completely negative. In contrast, peak cytotoxicity was obtained in the presence of a wide range of Ly 2.1-treated cells. Positive cytoxicity, although less than optimal, was also obtained in the presence of anti-Ly 1.1-treated cells ($10\text{-}30x10^3$). The mixture control behaved exactly like the Ly 2.1-treated cells. These results can be interpreted to suggest that the presence Ly 1.1^+, Ly 2.1^+ of inhibitory cells in the NMS-treated first step cultures is preventing the expression of help in second-step culture. Consequently, a negative cytotoxic response is observed. When these inhibitory cells are removed by treatment with either anti-Ly 1.1 or anti-Ly 2.1 antisera, the cytotoxic

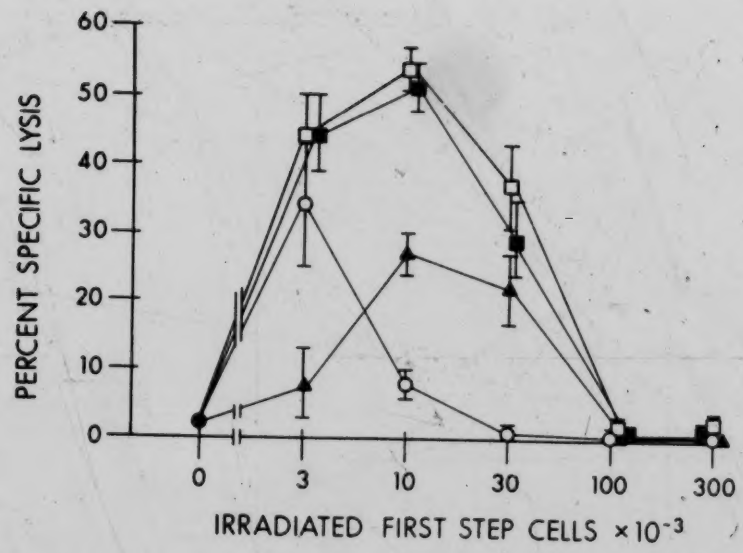

Figure 4. Helper T cells bear Ly 1.1 but not Ly 2.1 antigens. Helper cells were generated in 3-day first-step cultures and then treated with various anti-Ly sera plus complement. Helper activity was assayed as the ability to help the response of $3x10^5$ CBA thymus cells to $5x10^5$ irradiated BALB spleen cells in second-step cultures. Numbers of first-step cells used were equivalent to the number of cells in NMS-treated populations. The first-step cells used here were aliquots of the populations assayed for inhibitory activity in the experiments reported in Figure 2.

● = no first-step cells cells

O = no first-step cells cells

▲ = anti-Ly 1.1-treated first-step cells

□ = anti-Ly 2.1-treated first-step cells

■ = mixture of anti-Ly 1.1 and Ly 2.1-treated cells

response becomes positive.

6. Examination of the Ly phenotype of the precursors of suppressor cells

Spleen cells from normal CBA mice were treated with NMS, anti-Ly 1.1 and anti-Ly 2.1 antisera and complement. Viable cell counts were determined and $4x10^6$ cells were stimulated by $8x10^6$ BALB/c irradiated spleen cells. After 3 days in culture the cells were harvested, irradiated and added as indicated to second step cultures to assay for their ability to suppress the induction of CTL. Second step cultures were prepared by mixing $3x10^5$ CBA spleen responder cells and $3x10^5$ irradiated BALB/c spleen stimulator cells. The data from a typical experiment is shown in Table VI. Cells treated with anti-Ly 1.1 antiserum prior to setting up first step cultures were unable to generate effective suppression. The suppressive effect of these cultures wanes at $1x10^5$ cells tested. In contrast, cells treated with NMS exerted a strong suppressive effect. As few as $3x10^4$ cells from these cultures were able to suppress the cytotoxic response. Treatment with anti-Ly 2.1 antiserum was also effective in removing the ability to generate suppressive activity in first step cultures. This effect, however, is less dramatic than that of treatment with anti-Ly 1.1 antiserum. A 3-fold reduction in the suppressive activity was observed after treatment with anti-Ly 2.1 whereas a 10-fold reduction was observed after treatment with anti-Ly

Table VI. Ly phenotype of precursors of suppressor and helper T cells.

Type of treatment of precursor cells(a)	% specific lysis Number of first step cells added				
	None	$3x10^5$	$1x10^5$	$3x10^4$	$1x10^4$	$3x10^3$
Suppressor assay(b)						
NMS	57±11	1±1	3±2	13±2	31±6	30±8
anti-Ly 1.1		11±3	41±12	37±6	53±7	56±8
anti-Ly 2.1		2±2	9±3	46±13	58±4	54±8
Mixture (c)		6±3	41±5	49±8	55±5	58±12
Helper assay(d)						
NMS	11±8	NT	0.0	0.0	0.0	12±4
anti-Ly 1.1		NT	12±4	33±13	44±17	68±8
anti-Ly 2.1		NT	1±2	12±10	22±12	63±12
Mixture (c)		NT	6±4	30±20	54±12	67±5

a) Cells from normal CBA spleen were treated with the appropriate anti-Ly antisera and complement. Viable counts were determent and $4x10^6$ cells were mixed with $8x10^6$ BALB/c irradiated spleen cells and cultured for 3 days.

Table VI cont'd.

b) Results of second step cultures prepared from $3x10^5$ CBA spleen responder cells and $3x10^5$ irradiated BALB/c spleen stimulator cells.

c) A mixture of anti-Ly 1.1-treated cells and anti-Ly 2.1-treated cells.

d) Results of second step cultures prepared with $3x10^5$ thymus responder cells from 5-week-old CBA mice and $3x10^5$ irradiated BALB/c spleen stimulator cells. All values represent the mean ± S.D. of 4-16 replicate cultures.

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1.1 antiserum. A mixture of cells from anti-Ly 1.1 and anti-Ly 2.1 populations, treated separately, was also included. The suppressive effect obtained from these cultures was comparable to that of the anti-Ly 1.1-treated cells. At first glance these results indicate that the precursors of suppressor cells bear both Ly 1 and Ly 2 markers. It is also possible that cells which are Ly 1^+, Ly 2^+ are required for the induction of suppressor cells. In the absence of such cells differentiation of suppressor cell precursors to effector suppressors does not take place and therefore suppressor cells would not be induced.

Similarly, inconclusive results were also obtained with respect to the Ly identity of the helper precursor cells. In the helper assay shown in Table VI the results indicate that, in spite of the cytotoxic treatment with the antisera,

significant helper activity was observed in all groups except that of the NMS control. This data could be interpreted that either the helper cell precursor does not bear any Ly markers or the treatment with anti-Ly 1 and anti-Ly 2 sera was effective in removing an inhibitory component the presence of which is demonstrated in the suppressor assay of the same experiment. This inhibitory cell could bear the Ly 1^+, 2^+ markers. This is hinted at by the behavior of the cells from the mixture control in comparison to the activity of the cells from the NMS control. This analysis further complicates the approach to identify the Ly markers on regulatory cells, in particular the helper cells, at the precursor cell level. These results are subject to further discussion in Chapter V.

7. Regulation at low antigen dose

In the above experiments, the regulatory activity generated in first step cultures was characterized under conditions where high numbers of stimulator cells ($3x10^5$ cells) were used in second step cultures. We then decided to examine the nature of this regulation in the presence of low numbers of stimulator cells. The cells (responders, stimulators and first step cells) used in this experiment are aliquots of the same pool of cells used in the experiments reported in Figures 2 and 4. Second step cultures were prepared by mixing $1x10^6$ responder thymus cells with $3x10^4$ stimulator cells. A graded number of cells

from first step cultures were added to test for their ability to help or suppress the cytotoxic response. The results are shown in Figure 5. Under these conditions, the cytotoxic response, in the absence of cells from first step culture, is completely negative. This response could not be helped by cells from first step culture treated with either NMS or anti-Ly 1.1 at any of the cell numbers tested. In contrast, a positive cytotoxic response was obtained in the presence of a wide range of cells from anti-Ly 2.1-treated first step cultures. A significantly positive cytotoxic response was also observed at the lowest dilution of first step cells ($3x10^3$) from the mixture control. When compared to the results shown in Figure 4, these results indicate that as the number of stimulator cells (antigen dose) decreases, the inhibitory effect of cells from NMS-treated first step cultures becomes a stronger and does not shift to help. This inhibitory activity is mediated by cells which are Ly 1.1^+, 2.1^+ and cells which are Ly 2.1^+ confirming the observations shown in Figures 2 and 4. Previously (67), we have shown that as the number of stimulator cells decreases, the net regulatory effect of cells from first step culture shifts from suppression to help. The results shown in Figure 5 are not consistent with this observation. This descrepancy lies in the fact that in the earlier studies, spleen cells from adult CBA mice were used as responder cells in second step culture whereas in Figure 5, thymus cells were used as responder cells in second step cultures. We decided to

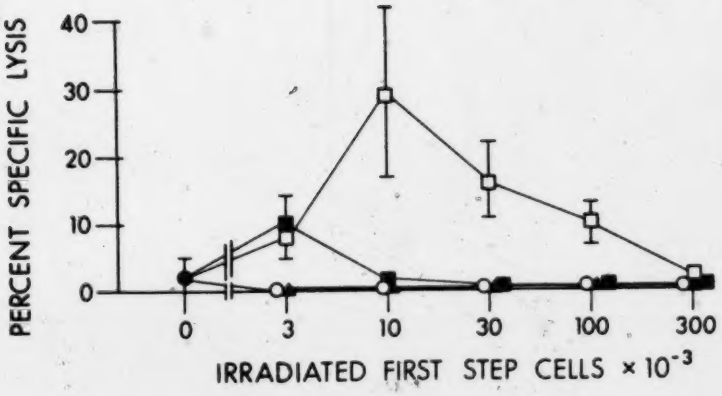

Figure 5. Regulation at low antigen dose. The regulatory cells used in this experiment are aliquots of the same population of first-step cells assayed for inhibitory and helper activity in the experiments reported in Figures 2 and 4. Graded numbers of these cells were added to second-step cultures which consist of $1x10^6$ thymus responder cells and $3x10^4$ irradiated BALB/c stimulator spleen cells. Results are shown as the arithmetic mean ± SE. This experiment has been repeated three times with consistent results.

• = NO first-step cells added

O = NMS

▲ = anti-Ly 1.1

□ = anti-Ly 2.1

■ = Mixture

further test this idea by examining the reproducibility of the regulatory pattern on thymus responder cells in the presence of varying numbers of stimulator cells. Second step cultures were prepared by mixing $3x10^5$ thymus responder cells with stimulator cells in the numbers indicated (Fig. 6). A wide range of cells from NMS-treated first step cultures were tested. The same first step cells were also treated with various anti-Ly sera, see legend of Figure 6. In the presence of high antigen dose ($5x10^5$ stimulator cells), the minimum number required to suppress the cytotoxic response is $1x10^5$ cells from first step cultures. At a lower antigen dose ($2x10^4$ cells), the minimum suppressive dose is $1x10^4$ cells from first step cultures. A medium range of stimulator ($1x10^5$) cells were also included. In this case, the minimum number required for suppression is $3x10^4$ cells. This number is exactly in the middle of the range of first step cells used to suppress the response at high and low antigen doses. In other words, the suppressive effect of first step cells is inversely related to the number of stimulator cells in second step cultures. This suggests that, in a system where thymus cells are used as responder cells in second step cultures, as the antigen dose decreases the suppressive effect of cells from first step cultures becomes more efficient and does not shift to help. This observation is in accord with data shown in Figure 5. Furthermore, the Ly phenotype of the inhibitory cells is Ly 1.1^+, 2.1^+, 5.1^+. These results are consistent with the

observation shown in Figure 2.

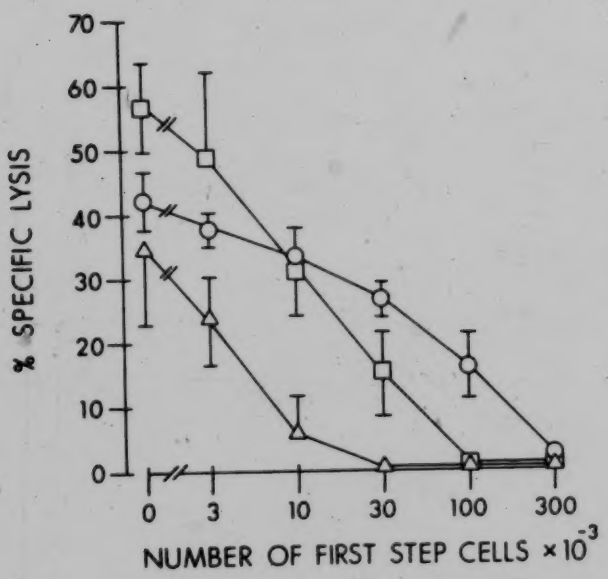

Figure 6. The effect of varying the antigen dose on the ability of cells from first step cultures to regulate the cytotoxic response by thymus responder cells. Second Step Cultures were prepared by mixing $3x10^5$ CBA thymus responder cells with irradiated BALB/c stimulator spleen cells in the numbers indicated O=$5x10^5$, □=$1x10^5$, Δ=$2x10^4$). To assay for suppression, graded numbers from NMS-treated 3 day first step cultures were added to second step cultures. Treatment with various anti-Ly antisera was also carried on the same first step cells. The results were as follows:

Figure 6 cont'd.

Type of treatment of first step cells	Viable cell counts $x10^{-6}$	% specific Lysis Number of Stimulator cells 5×10^5	1×10^5	2×10^4
		(42 ± 5)	(56 ± 7)	(35 ± 10)
NMS	4.2	16 ± 5	15 ± 6	5 ± 3
anti-Ly 1.1	2.0	33 ± 3	33 ± 4	16 ± 3
anti-Ly 2.1	1.4	29 ± 4	56 ± 7	40 ± 10
Mixture a) cells		27 ± 4	41 ± 4	38 ± 4
anti-Ly 5.1	0.5	51 ± 3	49 ± 5	31 ± 10
(number of first step cells tested)		(10^5)	(3×10^4)	(10^4)

Values represent the arithmetic mean ± S.E.

(a) A mixture of cells from anti-Ly 1.1 and anti-Ly 2.1 separately treated populations.

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8. The distribution of Ly 6 and Ly 7 on the suppressor and the helper cells

CBA anti-BALB/c cells from 3 day first step cultures were treated with the appropriate anti-Ly antisera and complement and NMS and complement. After treatment the cells were added to second step cultures prepared with CBA cells and BALB/c irradiated spleen cells. The data in Table VII show that the inhibitory cells are sensitive to treatment with anti-Ly 6.1. The degree of sensitivity to treatment with anti-Ly 6.1 varied depending on the type of the responder cell used in the second step culture. In the case of spleen responder cells the suppressive effect of anti-Ly 6.1 treated cells was 3-fold less than that of the NMS control; whereas in the case of thymus responder cells the suppressive effect was only slightly affected. This could be due to the fact that the overall suppressive effect in this experiment is relatively weak. As high as $1x10^5$ cells were required to observe an inhibitory effect on splenic responder cells. In both cases treatment with anti-Ly 7.2 has no effects. In order to establish whether or not the suppressor cells bear Ly-6.1 markers, the experiment was repeated with the inclusion of treatment with anti-Ly 1.1 antisera. As shown above treatment with anti-Ly 1.1 strongly reduced the suppressive effect of first step cultures. (Figures 1 and 2). A comparison, therefore, of the effect of anti-Ly 6.1 with the effect of anti-Ly 1.1 on the

Table VII. Treatment of suppressor cells with anti-Ly 6.1 and with anti-Ly 7.2.

		% specific lysis # first step cells added x10^{-5}			
Treatment of first step cells	None	30	10	3	1
Suppression of a response by spleen cells:					
NMS	64±4	3	7±1	43±8	66±9
anti-Ly 6.1		5±2	33±6	54±7	65±5
anti-Ly 7.2		5±3	10±4	44±8	51±8
Suppression of a response by thumus cells:					
NMS	64±5	1±2	0	27±19	48±13
anti-Ly 6.1		2±1	10±4	34±21	67±6
anti-Ly 7.2		2	1	4±2	23±23

3x10^5 CBA spleen cells or 10x10^5 CBA thymus cells were cultured with stimulator cells and the indicated number and kind of three day first step cells. Each group consisted of 4-12 replicate cultures: the values above represent the mean ± SD of replicate cultures. The large SD in the case of thymic responders (lines 6 and 8) plus first step cells are an indication that in these groups some cultures are suppressed while others are less affected.

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suppressive activity of first step cultures allows for a ctitical assessment of the expression of the Ly 6.1 marker by the suppressor cells. Again, cells from either spleen or thymus were used as responder cells in second step cultures (Table VIII). The results are similar to those reported in Table VII. Treatment with anti-Ly 1.1 effectively reduced the suppressive activity, approximately 10-fold, on both splenic and thymic second step cultures (compare the values of NMS at $3x10^4$ and anti-Ly 1.1 at $3x10^5$ first step cells added). When compared to these results, treatment with anti-Ly 6.1 was only slightly effective in reducing the suppressive activity on splenic second step cultures and has no or little effect on thymic second step cultures. A mixture of cells from anti-Ly 1.1 and anti-Ly 6.1 separately treated populations of first step cells was also included. The suppressive activity of this mixture was comparable to that of the NMS control. Taken together these observations show that while 3 day suppressor cells are rich in the expression of Ly 1.1 marker they are relatively poor in the expression of Ly 6.1 marker. Helper cells from similar cultures express the phenotype Ly 6.1^- and Ly 7.2^+ (91).

9. The Ly phenotype of cytotoxic T cells

We have demonstrated that the inhibitory cells generated in this system are different from the killer cells by several criteria (67). The next point to be examined is whether or not we could draw further support for this

Table VIII. Comparison of the effects of anti-Ly 1.1 treatment with those of anti-Ly 6.1 treatment of suppressor cells.

		% specific lysis # first step cells added x 10^{-5}				
Treatment of first step cells	None	30	10	3	1	0.3
Splenic Responder cells						
NMS	62±2	2	5±5	23±6	39±3	50±5
anti-Ly 1.1		18±8	36±13	56±9	49±10	71±17
anti-Ly 6.1		6±2	18±5	39±3	49±10	59±5
Mixture		1	5±4	36±4	49±15	58±8
Thymic Responder Cells						
NMS	17±6	1	0	0	2±2	11±3
anti-Ly 1.1		0	2±2	6±3	17±8	20±20
anti-Ly 6.1		0	0	2±2	6±1	17±9
Mixture		0	0	0	3±2	10±2

See legend to Table VII for culture conditions. Three day first step cells were used. The mixtures consisted of equal parts of anti-Ly 1.1 and anti-Ly 6.1 treated cells. Viability after treatment: NMS = $4x10^6$ cells; anti-Ly 1.1 = $2.5x\ 10^6$ cells; anti-Ly 6.1 = $1.5x10^6$ cells.

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hypothesis on the basis of typing for Ly antigens. After 5 days of incubation, killer cells from cultures of CBA spleen cells responding to BALB/c stimulator cells were treated with various anti-Ly sera and complement and assayed for their cytotoxic activity on ^{51}Cr-labeled P815 target cells. Figure 7 shows that the killer cells are sensitive to treatment with anti-Ly 2.1. The cytotoxic activity was significantly reduced from that of the NMS control. In contrast, treatment with anti-Ly 1.1 (#458) has no effect on the cytotoxic response. Nevertheless, these results were not true of all the experiments which were done in this study. Results obtained from experiments where a different batch of anti-Ly 1.1 serum (#466) was used gave different conclusions. The results shown in Table IX indicate that with this batch of serum, killer cells were also sensitive to treatment with anti-Ly 1.1. A 3-fold reduction (from the NMS control) in the killer activity was observed after treatment with either anti-Ly 1 or anti-Ly 2. To determine whether the decrease in the killer activity was due to depletion of one subset of killer cells which bear both Ly-1 and Ly-2 antigens or two subsets of killer cells, one bearing Ly-1 antigen and the other bearing the Ly-2 antigens, a mixture control composed of anti-Ly-1-treated cells and anti-Ly-2-treated cells was thus included in the essay. The results indicate that the lytic activity obtained from the mixture control was not comparable to the NMS control. This suggests that the killer cells are one subset

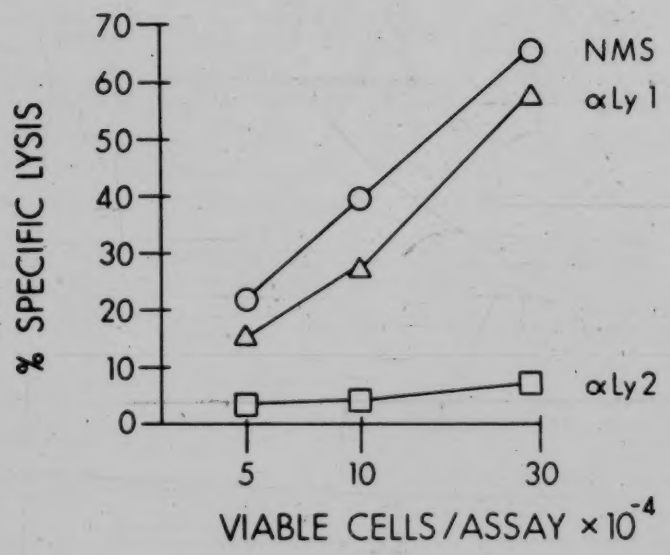

Figure 7. Ly phenotype of killer cells. CBA responder spleen cells were cultured with irradiated BALB/c stimulator cells. After five days in culture, effector cells were treated with various anti-Ly antisera and tested for their ability to lyse ^{51}Cr-labeled P815 target cells. The results are plotted in per cent specific lysis, mean ± S.D. of three replicates. Viable cell recovery was: NMS=5.5x10⁶/ml, anti-Ly 1.1=1x10⁶/ml, anti-Ly 2.1=1x10⁶/ml. These results were obtained after treatment with anti-Ly 1.1 #458. Spontaneous release: 855 ± 25 cpm; detergent release: 8704 ± 916.

o = NMS

▲ = anti-Ly 1.1

□ = anti-Ly 2.1

of T cells which bear both the Ly 1.1 and the Ly 2.1 markers. (This discrepancy in the Ly phenotype of the killer cells will be further analyzed in the discussion.

In order to assess directly the relationship between cytotoxic cells and suppressor cells, we decided to assay the cells from the same 5 day first step cultures for cytotoxicity and suppression in one experiment. After treatment with the appropriate antiserum (including anti-Ly 1.1 #466), cells from CBA anti-BALB/c first step cultures were divided into two aliquots. One aliquot was assayed for cytotoxicity on ^{51}Cr-labeled P815 target cells and graded numbers of cells from the second aliquot were added to second step cultures to assay for their ability to suppress the generation of CTL. The results from both assays are shown in Figure 3. Figures 3.a and 3.b represent data from two independent experiments. As indicated earlier, the inhibitory cells are Ly 1.1^-, 2.1^+. In contrast, the cytotoxic cells are Ly 1.1^+, 2.1^+ confirming the results shown in Table IX. Furthermore, there is no correlation between the level of cytotoxicity and the extent of suppression. For example, although the cytotoxic activity was either minimal or virtually removed at $3x10^4$ cells from NMS, anti-Ly 1.1 and anti-Ly 2.1-treated populations, only the suppressive influence of cells from NMS and anti-Ly 1.1-treated population is completely effective.

Table IX. Ly phenotypes of cytotoxic T cells using a second batch of Ly antisera.

Type of treatment	Viability after treatment cells x 10^{-6}	% specific lysis $1x10^5$	$1x10^4$
NMS	5.5	67 ± 3	11 ± 0
anti-Ly 1.1	2.0	18 ± 1	2 ± 0
anti-Ly 2.1	1.8	19 ± 0	3 ± 0
Mixture of Ly-1 treated cells and Ly-2 treated cells		28 ± 1	3 ± 0

CBA spleen responder cells were cultured with irradiated BALB/c spleen stimulator cells for five days. Cytotoxic cells were treated with the appropriate alloantisera and complement and the viable cells were assayed for cytotoxicity.

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10. The Ly 5 typing of suppressor cells, helper cells and killer cells

The Ly 5 locus has been identified only recently (34). It appears to control antigenic specificities which are restricted to T lymphocytes. We include here a summary of the results on the Ly 5 phenotype of killer cells, inhibitory cells and helper cells (Table X). The killer, helper and 5-day inhibitory cells were obtained from cultures of CBA spleen responder cells and BALB/c irradiated stimulator spleen cells. Inhibitory cells from 3 day first step cultures are also included. Cells were treated with the appropriate antisera and complement and assayed for the relevant function. When compared to NMS-treated cells, only residual cytotoxic activity was observed from anti-Ly 5-treated population. This indicates that the killer cells are Ly 5^+. Helper cells and inhibitory cells were added to second step cultures consisting of CBA thymus responder cells and BALB/c stimulator spleen cells. In the helper cell assay, helper activity by cells from NMS-treated population was observed at the lowest cell number tested ($3x10^3$). In contrast, no helper activity was observed by cells from anit-Ly-5-treated cultures. Similarly, the inhibitory activity obtained from 3-day first step cultures was also removed by treatment with anti-Ly 5 alloantisera. These results indicate that killer cells, inhibitory cells and helper cells all bear the Ly 5 marker.

Table X. The Ly 5 phenotype of killer cells, helper cells and inhibitory cells

Number of cells from first-step cultures	cytotoxic response[a] (% specific lysis)							
	Killer cell assay		Helper cell assay		Inhibitory cell assay (b)		(c)	
	NMS	anti-Ly 5	NMS	anti-Ly 5	NMS	anti-Ly 5	NMS	anti-Ly 5
None			4±		57±		42±	
$3x10^5$	56[d]	15	ND	ND	0	40.5	2	58
$1x10^5$	27	4	2	0	1.8	70	16	51
$3x10^4$	8	4	2	0	47.1	64	27	43
$1x10^4$	1	1	6	0	79	76	33	37
$3x10^3$	ND	ND	25±	1	75	82	ND	ND
viable cells recovered after treatment x 10^{-6}	6	1.5					4.2	0.5

(a) The cytotoxic response of second-step cultures consisting of CBA thymus responder cells ($1x10^6$) and BALB/c stimulator spleen cells. CBA anti-BALB/c first-step cells were treated with either NMS or anti-Ly 5 and added, in the numbers indicated, to second-step cultures to assay for either help or suppression.

(b) The cells were obtained from 5-day first-step cultures.

(c) The cells were obtained from 3-day first-step cultures.

(d) The results of the killer cell assay, helper cell assay and column c of the inhibitory cell assay were obtained from the same experiment. Each number represents the arithmatic mean of four replicate cultures.

11. Ia and IJ typing of suppressor, helper and killer cells

First-step cells from 3-day culture were treated with various dilutions of anti-Ia sera and complement and added to a second MLC to assay for their ability to inhibit the generation of CTL. The data show that the inhibitory activity was not removed by treatment with anti-Ia at any of the serum dilutions tested (Table XI). At no time did we observe any significant difference in the lytic activity between the anti-Ia-treated groups and the control groups. We repeated this experiment six times and all the results are consistent with these findings, that is, the inhibitory cells are Ia-negative. Similarly, in a helper cell assay, we found that the helper activity was completely unaffected by treatment with anti-Ia (92).

The killer activity was also unaffected by treatment with anti-Ia sera. Table XII shows that the lytic activity obtained from anti-Ia-treated cells which have been cultured for 5 days *in vitro* is not different from that of the NMS control. This indicates that the killer cells produced in this system are Ia-negative.

The IJ typing was negative for the killer cells and the 5-day inhibitory cells (Figure 3). Three-day inhibitory cells were also unaffected by treatment with anti-IJ (Table XIII).

Table XI. The Ia phenotype of suppressor cells generated in 3 day first-step culture.

Type of treatment	Number of step-1 cells added			Viable cell recovery x 10^{-6}/ml
	$1x10^6$	$5x10^5$	$2.5x10^5$	
NMS 1/4	5	10	22	5.4
1/16	11	14	32	4.8
Ia 1/4	7	18	25	1.8
1/8	7	18	24	1.8
1/16	5	13	19	2.5

Cells from 3-day first step cultures were treated with various dilutions of anti-Ia serum and C. After treatments, viable counts were determined and the cells were added, in the numbers indicated, to second step cultures to assay for suppression. The response of second step cultures where no cells from first-step cultures were added was 37% lysis.

Table XII. Ia phenotype of cytotoxic T cells.

Fraction of a culture	% specific lysis		
	Untreated	NMS-treated	anti-Ia -treated
1/10	30	23	33
1/30	16	13	16
1/60	9	8	4

Cytotoxic cells were harvested from 5-day cultures containing CBA spleen cells plus irradiated BALB/c spleen cells. Assay was on $1x10^5$ ^{51}Cr P815 target cells. Viability after treatment was as follows: untreated = $10x10^6$ cells, NMS-treated = $8.2x10^6$ cells, anti-Ia -treated = $5.6x10^6$ cells.

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Table XIII. Effect of treatment with anti-IJ on regulatory cells 3-day first-step cultures.

First step cells x 10^{-3}	% specific lysis			
	Inhibitory activity		Helper activity	
	NMS	anti-IJ	NMS	anti-IJ
None	25±2		5±2	
300	3 ± 1	0	10 ± 2	9 ± 3
100	10 ± 2	2 ± 1	14 ± 2	27 ± 5
30	19 ± 2	7 ± 2	24 ± 4	39 ± 2
10	26 ± 3	14 ± 2	20 ± 4	39 ± 4
3	25 ± 2	14 ± 4	17 ± 5	52 ± 3

Cells from first step cultures were treated with either NMS or anti-IJ(k) and complement. Viable cells recovered after treatment were: NMS = $7.5x10^6$, anti-IJ = $6.5x10^6$. The cells then were added, in the numbers indicated, to second step cultures to assay for their suppressor or helper activity. The inhibitory assay consists of $3x10^5$ CBA spleen responder cells and $3x10^5$ irradiated BALB/c stimulator spleen cells. The helper assay consists of $1x10^5$ CBA responder cells and $3x10^5$ irradiated BALB/c stimulator spleen cells. Values represent the mean ± SE of 8-40 replicate cultures.

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CHAPTER IV

Helper, suppressor and cytotoxic cells are physically distinct subclasses of T cells

A. Discussion

1. Is suppression due to antigen elimination?

The data shown in Table III indicates that there is an inverse relationship between the number of stimulator cells used in the second step culture, and the effect of irradiated first-step cells on that culture. In the presence of high numbers of stimulator cells a particular number of first-step cells ($1x10^5$) is suppressive, whereas in the presence of a 50-fold lower number of stimulator cells, the same number of first-step cells actually helped the response. This observation speaks strongly against antigen elimination as the major mechanism underlying the suppressive effect of first step culture on the generation of cytotoxicity.

Alternatively, it is possible that the cytotoxic effect is directed against responder cells which have stimulator cell alloantigens bound to their surface, thus rendering them susceptible to the cytotoxic activity of first-step cells. The data reported in Table III also speaks against this possibility. A three-fold reduction in the number of responder cells did not seem to influence the suppressive effect of first-step cells. That is, the relationship

between the antigen dose and the effect of first-step cells was maintained regardless of the decrease in the number of responder cells present in second step culture. Our interpretation of this data suggests that the suppressive effect is not due to cytotoxic effect, but rather is a regulatory signal which occurs as a result of the ability of both responder cells and inhibitory cells to recognize and respond to determinants expressed on the simulator cell surface. This interpretation is also consistent with the experiments reported by Truitt et al (29), who demonstrated that increasing the number of stimulator cells in second step cultures did not competitively diminish the suppressor activity.

Specific cytotoxic interactions involving only responder cells and first-step cells which occur via alloantigen bound to the surface of either cell type are unlikely, due to competitve interactions which must occur in the presence of large numbers of stimulator cells. This is justified on the grounds that the number of responder cells specific for BALB/c is at least 10^3-fold lower than the number of BALB/c stimulator cells based on the results of others (92). Anti-BALB/c-specific interactions are most likely to occur between responder cells and stimulator cells, and between first-step cells and stimulator cells, thereby preventing any direct cytotoxic interactions between first-step cells and responder cells.

In view of this discussion, it is worth emphasizing

that the level of cytotoxicity is defined by the lysis of ^{51}Cr-labeled P815 target cells in a 4-6h assay. Since irradiated fresh BALB/c spleen cells are used as stimulators, and in general, these are very poor targets in the 4-6h assay for cytotoxic T cells, it is somewhat speculative to assume that the suppressive effect of first step culture is due to elimination of stimulator cells in the second culture. However, if we accept the fact that we are somewhat limited in the means by which cytotoxic activity is defined (e.g. the ^{51}Cr release assay), one might argue that cytotoxic activity which is undetectable by conventional methods is responsible for the inhibitory effects observed here. This is unlikely for a number of reasons. First, a 50-fold reduction in the stimulator cell number does not eliminate the cytotoxic response of uninhibited second step culture (Table III, $5x10^6$ to $1x10^5$ stimulator cells). However, cells from first step culture still able to very efficiently inhibit the cytotoxic response to $5x10^6$ stimulator cells. Second, a 100-fold decrease in stimulator cell number does in fact severely reduce the cytotoxic response, but under these conditions, the first-step cells help rather than inhibit the cytotoxic response. Thus, it becomes very difficult to support an argument which is based on undetectable cytotoxic effects by cells from first step cultures.

2. Is suppression "too much" help?

The observation that a certain number of irradiated first-step cells can inhibit the generation of CTL in the presence of high numbers of stimulator cells, and that this same number of first-step cells can help the cytotoxic response in the presence of a 5-fold lower dose of stimulator cells, requires further discussion (Table III). These results could be interpreted as reflecting one of two broad possibilities: either suppressor cells cannot function properly in the presence of low doses of antigen, or suppressor and helper functions are mediated by the same type of T cell. Observations on the generation in vitro of helper T cell activity for the induction of CTL seems to support the second possibility. These results show that helper activity is generated in first step culture harvested at days 2 and 3. The helper activity of day 3 first step culture (which are equivalent to the first step culture described here) is 10 to 20-fold higher than the helper activity in first step culture harvested at day 2. This correlates well with the increased inhbitory activity described here which is also seen in day 3 first step culture. However, the postulate that the inhibitory effect of first-step cells represents excess helper activity is more difficult to reconcile with the observation that pretreatment of mice with cortisone seems to remove precursors of the inhibitory cells while apparently enriching for precursors of helper activity (67).

Another approach for a better understanding of the relationship between helper cells and suppressor cells and the relationship between suppressor cells and cytotoxic cells is described below.

The detection and the nature of distribution of Ly determinants on regulatory cells provide new insights into the role and mechanism of action of inhibitory and helper cells in immunoregulation. The series of experiments described in Chapter 3 have demonstrated that T helper cells are clearly different from T inhibitory cells; whereas the helper cells bear Ly 1.1 but not the Ly 2.1 marker, the inhibitory cells bear both Ly 1.1 and 2.1 markers. A second type of inhibitory T cell which bears the Ly 2.1, but not the Ly 1.1 marker can also be identified after five days of culture, but is only sporadically detected in three-day first-step cultures. Furthermore, the suppressor cells are physically distinct from the cytotoxic T cells. These experiments were done using cell populations in which helper cells, suppressor cells and killer cells coexist and can be assayed independently. All of these studies refer to the surface phenotype of the effector cells whose function is radioresistant.

The analysis of the Ly surface phenotype of effector cells is a much more straight forward experimental problem than is the analysis of precursor cell phenotype. If a radioresistant regulatory cell function is being assayed, inductive events have occurred prior to treatment with

various antisera. The probability that the cell type removed by treatment represents the actual effector cell of a given function is high. If precursor cells are treated, it becomes difficult to ensure that all the elements necessary for induction of those precursors are unaffected by the treatment. For example removal of a precursor function by anti-Ly 1 treatment might not mean that the precursor itself bears the Ly 1 marker.

To illustrate this problem the data presented in Table VI offers a good example. The data failed to support the conclusion on the nature of the Ly phenotype of either the suppressor or the helper cell precursor. This uncertainty stems from the fact that, given the nature and the method of the assay system, one can never achieve full isolation and independant characterization of the precursors of a certain subclass of T cells without the influence of the other subclasses. For example, if one were to treat a population of cells from normal spleen with Ly 1 antisera and achieve only a 3-fold reduction in the number of helper cell precursor and a 10-fold reduction of the inhibitory cell precursor, it is likely that, under these conditions, the helper activity generated from anti-Ly treated spleen would be better than the helper activity of the NMS control where the precursor of inhibitory cells were not affected. Consequently, the helper activity obtained from these first-step cultures would result in a strong cytotoxic response in the second-step culture. These results are

misleading. The fact that we got high level of helper activity in the first-step cultures is not because the helper cell precursors do not bear Ly 1 but because in the absence of 10-fold or higher of inhibitory cell precursors, the helper cell precursor are able to proliferate uncontrolled.

The complexity of the interpretation of such data could be extended to other systems as well [Bach and Alter (11), Swain and Panfili (42) and Cantor and Boyse (41)].

The presence of two different subsets of inhibitory T cells in 3-day first-step culture raises the question of whether or not these cells represent a single differentiation pathway. Cantor and Boyse postulated that Ly 1^+2^+ cells could be the common precursors for the other subclasses of T cells characterized as Ly 1^-, Ly 2^+ and Ly $1^+,2^-$ (43). This could mean that the Ly $1^+,2^+$ subclass of inhibitory cells at 3 days of culture is transitional and will further differentiate to yield Ly 2^+ progeny although it is important to realize that the 3-day Ly $1^+,2^+$ suppressor is a differentiated cell capable of exerting a radioresistant inhibitory function. This model predicts that the majority of inhibitory cells from first step cultures of longer period of incubation (for example, 5 days) should predominantly express the Ly 2 marker. This was shown to be the case (Figure 2). A second model would predict that Ly $1^+,2^+$ and Ly $1^-,2^+$ cells are separately differentiated regulatory cells, each of which is capable of exerting an

independent inhibitory effect. The studies on precursor phenotype described in Chapter III were designed to test this model but, as discussed, have proven difficult to interpret.

The mechanism by which these inhibitory cells act is an interesting problem. Some of these data can be interpreted as suggesting that the Ly 1$^-$,2$^+$ inhibitory cell may have a different mechanism of action than does the Ly 1$^+$,2$^+$ inhibitory cell. This is hinted at in the data presented in Figures 2 and 4. Both types of suppressor cells inhibit the generation of CTL at the highest antigen dose tested, and the Ly 1$^+$,2$^+$ inhibitor is about 3-fold more frequent than the Ly 1$^-$,2$^+$ inhibitor (Figure 2). The suppressor activity of the same population of cells when assayed at low antigen dose (Figure 5) seems to be mediated largely by Ly 1$^-$,2$^+$ inhibitory cells. This can be interpreted as indicating that at low antigen dose, induction of CTL is much more dependent on the generation of helper cells from within the second step culture than is the case at higher antigen dose. Under these conditions it is possible that the Ly 1$^-$,2$^+$ suppressor is a more efficient inhibitor of the induction of help than is the Ly 1$^+$,2$^+$ suppressor.

It seems clear that in operational terms, the Ly 1$^+$,2$^+$ suppressor cell acts to negate the activity of helper cells. In Figure 4, using the same population of first step cells as in Figures 2 and 5, it was necessary to dilute the first-step cells to 3×10^3 cells before helper activity could

be detected, suggesting that at higher cell doses the activity of helper cells was obliterated by inhibitory effects. Treatment of the first step cells with anti-Ly 1.1 depleted the helper activity approximately 3-fold and must also have removed some of the inhibitory component since now help is seen at doses of first-step cells which were not effective if untreated. Treatment with anti-Ly 2.1 dramatically increased the helper activity and also broadened by 10-fold the range over which helper activity was seen. This indicates that an inhibitor bearing Ly 2 antigens prevented the expression of helper activity. The mixture of anti-Ly 1.1 and anti-Ly 2.1-treated cells shows that the inhibitor which acts under these circumstances is a Ly $1^+,2^+$ cell. This part of the experiment shows no trace of the Ly $1^-,2^+$ inhibitor which, from the data of Figures 2 and 4, we know was present in the first step cell population. In terms of the inhibition of the induction of CTL precursors, the Ly $1^+,2^+$ inhibitor appears to be the major suppressive activity. These experiments make clear the complications of interpreting data using anti-Ly sera even at the effector cell level, and also indicate the importance of testing treated populations at multiple cell doses to assess the degree of effect of any given serum.

The results on the relationship between the regulatory effect of cells from first step cultures on the generation of CTL and the number of stimulator cells are most intriguing. Firstly, at low antigen dose, more help is

required for the generation of a positive cytotoxic response. This is evident from the fact that, at high antigen dose, the helper activity by as few as $3x10^4$ cells from anti-Ly 2.1-treated population was effective in helping the cytotoxic response (Figure 4). In contrast, at low antigen dose, this number was not effective; in fact, a 3-fold higher number ($1x10^5$) of cells from the anti-Ly 2-treated first step cells was needed to provide effective help for the generation of CTL (Fig. 5). Secondly, as the number of stimulator cells decreases, the suppressive effect of cells from first step cultures becomes stronger and does not shift to help. In the presence of $3x10^5$ stimulator cells, the cytotoxic response is completely inhibited by $3x10^4$ cells from NMS-treated population (Fig. 2). A 10-fold reduction in the number of stimulator cells the cytotoxic response is strongly suppressed by as low as $3x10^3$ cells from the same pool of NMS-treated cultures used in Figure 2 (Fig. 5). Furthermore, the helper activity by $3x10^3$ cells from NMS-treated cultures observed at high antigen dose in Figure 4 is completely absent at low antigen dose (Fig. 5). In other words, as the antigen dose decreases, the suppressive effect of cells from first-step cultures becomes more efficient. The same results were also obtained in another independently designed experiment (Fig. 6). These results are in contrast to the data reported in Table III. Using splenic responder cells, we observed that as the antigen dose decreased, the net regulatory effect of cells

from 3-day first-step cultures shifted from suppression to help. This discrepancy could be interpreted to suggest that the nature of regulation for the generation of CTL from spleen responder cells is different from that of thymus responder cells. Further investigation of this phenomenon will hopefully provide some insight into the mechanism of T-T cell interactions in the induction of cytotoxicity.

The discrepancy in the results of the Ly phenotype of the killer cells requires further discussion. Two different batches of anti-Ly 1.1 serum were used in most of these experiments. It is relevant to emphasize here that when any batch of the Ly serum was used to type a certain population of cells from first-step culture, the same cells were used in three ways; in the helper assay, the suppressor assay and the killer assay in one experiment. Throughout all these experiments, using the two batches of anti-Ly 1.1 sera, consistent results were always obtained with regard to the Ly phenotype of the regulatory cells. The helper cells were Ly $1^+,2^-$ and the inhibitory cells from 3-day first-step culture were Ly $1^+,2^+$. Table XIV provides a summary of the experiments done with various batches of serum. Only the Ly phenotype of the killer cells differ from one batch of sera to the other. The results from the first batch of anti-Ly 1.1 (#458) show that the killer cells are Ly 1^-2^+. In contrast, the results from the second batch (#466) show that the killer cells are Ly 1^+, 2^+. This difference could be a result of either or both of two factors: (1) an antibody

specificity present in anti-Ly 1.1 #466 but not in anti-Ly 1.1 #458, or (2) a change in the nature of the Ly antigens expressed on the surface of the killer cells. We cannot substantiate with certainty either of these possibilities. At best, this discrepancy demonstrates some of the difficulties which are frequently encountered when working with serological reagents such as the Ly antisera. However, this observation does not disqualify our previously made conclusion (67) that, on the basis of several critera (for example, cortisone sensitivity), the inhibitory cells detected from 3-day first-step culture are different from cytotoxic T cell. In fact, the results of the Ly typing reported here are in accord with this hypothesis. This is evident from two main observations: (1) When the first batch of serum was used, the inhibitory cells from 3-day first step culture were Ly 1^+, 2^+ and the killer cells from 5-day cultures were Ly 1^-, 2^+; (2) when the second batch of serum was used, the inhibitory cells from 5-day culture were Ly 1^-, 2^+ and the killer cells from the same culture were Ly 1^+, 2^+. These experiments indicate that, on the basis of cell surface phenotype, killer cells are physically distinct from suppressor cells.

This is not the only system in which Ly 1^+2^+ cytotoxic T cells have been shown. Beverly *et al.* (55) showed that killer cells obtained from CBA (H-2k, Ly 1.1, Ly 2.1) mice are Ly 1^+2^+. Shiku *et al.* (54) reported that while killer cells from C57B1/6 (H-2b, Ly 1.2, Ly 2.2) mice are Ly 1^-2^+,

Table XIV. Summary of Ly markers on effector cell types involved in cytotoxic responses.

	Ly phenotype (# experiments)	
	Serum batch #1	Serum batch #2
Cytotoxic T cells at 5 days	Ly 1.1⁻ Ly 2.1⁺ (2)	Ly 1.1⁺ Ly 2.1⁺ (4)
Suppressor T cells at 3 days	Ly 1.1⁺ Ly 2.1⁺ (4) Ly 1.1⁻ Ly 2.1⁺ (1)	Ly 1.1⁺ Ly 2.1⁺ (2)
Suppressor T cells at 5 days	———	Ly 1.1⁻ Ly 2.1⁺ (2)
Helper T cells at 3 days and 5 days	Ly 1.1⁺ Ly 2.1⁻ (2)	Ly 1.1⁺ Ly 2.1⁻ (1) (a) Ly 1.1? Ly 2.1⁻ (2) Ly 1.1⁺ (3)

Serum batch #1 included anti-serum #458 (anti-Ly 1.1) and anti-serum #714 (anti-Ly 2.1).

Serum batch #2 included anti-serum #466 (anti-Ly 1.1) anti-serum #744 (anti-Ly 2.1), and anti-serum #927 (anti-Ly 2.1).

All of the above cell types at both points in time were Ia⁻ and Ly 5.1⁺.

None of the cell functions assayed above were affected by treatment with anti-IJ.

(a) Due to puzzling results with the mixture controls, we have two experiments in which the Ly 1 phenotype of the helper cell cannot be definitely shown to be Ly 1⁺, but these same experiments clearly show it to be Ly 2.1⁻.

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the killer cells from C57B1 Ly 1.1 congenic mice are Ly 1+2+. Results very similar to ours using the same batches of serum were obtained by Shaw et al. (93) in an analysis of the CTL precursor.

The expression of the Ly 5.1, the Ly 6.1 and the Ly 7.2 markers on the effector cells of suppression, help activity and cytotoxicity has also been examined. Although all of these effector cells express the Ly 5.1 allele they greatly differ in the expression of the Ly 6.1 and the Ly 7.2 alleles. The helper cells express the phenotype Ly 6.1−, Ly 7.2+, the suppressor cells express the phenotype Ly 6.1+?, Ly 7.2− and only 50% of the cytotoxic cells express the phenotype Ly 6.1+, Ly 7.2− (91). The question mark indicates the uncertainty of the evidence. This uncertainty stems from the following observation: The evidence on the expression of the Ly 6.1 allele by the suppressor cells varied depending on the nature of the responder cells employed in the second step cultures (see results: Tables VII and VIII). This probably does not directly reflect on the problem whether or not suppressor cells bear the Ly 6.1 marker. This is true especially in view of the fact that thymus responder cells are more susciptible to the suppressive effects of first step cells than splenic responder cells. (Figure 5 and 6). This observation, however, suggests that either there are two populations of suppressor cells one population expresses the Ly 6.1 marker and the other does not or there is one population of suppressor cells which is relatively poor in

the expression of the Ly 6.1 marker. The observation that the treatment with anti-Ly 6.1 was only 30% effective in removing the suppressive activity whereas treatment with anti-Ly 1.1 was 90% effective is consistent with the latter explanation.

The possibility that there are two suppressor cells one expresses the Ly 6.1 marker and the other does not is consistent with the data presented earlier in this volume. Namely that one suppressor cells expresses the phenotype Ly 1.1+, Ly 2.1+ and the other expresses the phenotype Ly 1.1−, Ly 2.1+. The question is which one of these suppressor cells expresses the Ly 6.1 marker? The observation that the helper cells which expresses the phenotype Ly 1.1+, Ly 2.1− is also Ly 6.1−, Ly 7.2+ hints at the possibility that the suppressor cells which express the Ly 1.1 marker could also be Ly 6.1−. Although genetic studies have ruled out any linkage relationship between the Ly 2.1 and Ly 6.1 alleles (37), a functional relationship between these two alleles is very likely.

In the next series of experiments, we wished to determine whether or not inhibitory cells differ from helper or killer cells in the expression of surface markers other than the Ly markers. Two anti-I region sera were tested, anti-Ia and anti-IJ. Our data show that all three subclasses of T cells (i.e. T helpers, T inhibitors and T killers) were Ia− and IJ−. These sera were tested at several dilutions. The cytotoxic titer of the anti-Ia serum was 1:50 and we

used it at a dilution of 1:4. Even at this level we could not detect any Ia determinants on any of the T cell inhibitors, helpers or killers.

The absence of I-region-coded determinants on the inhibitory cells in our system is in contrast to reports published previously. Tada et al. showed that the suppressive T cell factor which regulates the antibody response to DNP-KLH is a product of the IJ subregion genes (82). Furthermore, Murphy et al. demonstrated that the allotype-specific suppressor cell is sensitive to treatment with anti-IJ antisera (83). This could imply that suppressor mechanisms which regulate humoral responses are different from those suppressor mechanisms which regulate the induction of CMI. However, recent evidence published by Greene et al. does report the presence of IJ determinants on a suppressor factor which inhibits contact sensitivity (94).

In conclusion, using specific alloantisera raised against T cell differentiation antigens, we were able to dissect the regulatory role of T cells in the induction of CTL and identify its components. It appears that the helper activity is mediated by a subset of T cells which selectively expresses Ly 1 determinants (Ly 1^+, 2^-). This is clearly different from the inhibitory T cells which at three days of culture express Ly 1 and Ly 2 (Ly 1^+, 2^+) determinants and at five days of culture selectively express Ly 2 (Ly 1^-, 2^+) antigens. Furthermore, cytotoxic cell effectors generated in this system also belong to a

physically distinct subclass of T cells.

B. Summary

Regulatory T cells can be obtained from primary mixed lymphocyte cultures of CBA spleen cells responding to BALB/c stimulators. At day three of culture, T cells are generated which can either help or suppress the generation of cytotoxic T cells in a second primmary MLC culture. The regulatory activity observed depends on the conditions employed in the assay system. Both the helper activity and the suppressor activity are mediated by differentiated antigen-specific T cells whose function is radioresistant. The Ly and Ia phenotype of these regulatory cells was tested. The phenotype of the helper cells is Ia^-, Ly 1.1^+, Ly 2.1^- and Ly 5.1^+ whereas the inhibitory cells are Ia^-, Ly 1.1^+, Ly 2.1^+ and Ly 5.1^+. At day 5 of MLC culture, suppressor activity and helper activity are also observed. However, at this point in the generation of suppressor cells, an inhibitor which is Ia^-, Ly 1.1^-, Ly 2.1^+ and Ly 5.1^+ represents the major inhibitory activity. These two types of suppressor cells may regulate the induction of cytotoxic T cells via different mechanisms based on experiments in which their activity is measured as a function of antigen dose.

The Ia and the Ly phenotype of the cytotoxic cells was also tested. Cytotoxic T cells express the phenotype Ia^-, Ly 1.1^-, Ly 2.1^+ and Ly 5.1^+. Using a second batch of anti-Ly

1.1 antisera the killer cells express the phenotype Ly 1.1$^+$ and Ly 2.1$^+$. However the inhibitory cells from the same culture expressed the phenotype Ly 1.1$^-$ and Ly 2.1$^+$. Taken together these observations show that the antigen-specific suppressor cells, helper cells and cytotoxic cells represent physically distinct subclasses of T cells.

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